3. Agitate the mixture rapidly by pumping it up and down twice in the pipette tip.
4. Allow to stand for 30 min at room temperature.

3.4 Evaluation of Transgene Expression in the Mouse

1. Administer 300 µl of lipoplexes containing appropriate amounts of DNA into a mouse via the appropriate route.
2. Six hours later, kill the mouse by cervical dislocation or perfusion with saline through the portal vein.
3. Excise the organs (spleen, liver, kidney, heart and lung) and wash them twice with ice-cold saline.
4. Weigh the excised organs and add an appropriate volume (5 µl/mg for liver and 4 µl/mg for other organs) of lysis buffer.
5. Homogenize the organs using a homogenizer.
6. Transfer 400 µl of homogenate into a microtube and perform three cycles of freezing (liquid N_2 for 3 min) and thawing (37°C for 3 min).
7. Centrifuge the homogenate at 10,000 × *g* for 10 min at 4°C.
8. Analyze the luciferase activity in 20 µl supernatant using a luminometer.
9. Determine the protein concentration of lysate using a Protein Quantification Kit.

3.5 Cellular Localization of Luciferase Activity in Liver

In order to reduce unexpected side effects, tissue- or site-specific targeted delivery of the exogenous therapeutic gene using a gene carrier is necessary for gene therapy. For this reason, many researchers select liver for the target tissue, because asialoglycoprotein and mannose receptors are specifically expressed on the surface of hepatocytes (liver parenchymal cell) and macrophages, such as Kupffer's cells (liver non-parenchymal cell), respectively. Therefore, it is important to evaluate whether the sugar modified gene carrier system is working correctly. In the following, a method to evaluate the hepatic cellular localization of luciferase activity by separating parenchymal and non-parenchymal cells is described.

3.5.1 Separation of Parenchymal and Non-parenchymal Cells from Liver

1. Anesthetize a mouse with an intraperitoneal injection of pentobarbital sodium (50 mg/kg) 6 h after the administration of lipoplexes.
2. Perfuse the liver with Ca^{2+}, Mg^{2+}-free perfusion buffer for 10 min at a perfusion rate of 3–4 ml/min. As soon as perfusion is started, cut the vena cava and aorta.
3. Perfuse the liver with perfusion buffer supplemented with Ca^{2+} and type I collagenase for a further 10 min. Again, a perfusion rate of 3–4 ml/min should be maintained.
4. Excise the liver and remove its capsular membrane.
5. Disperse the liver cells into ice-cold Hank's-HEPES buffer containing 0.1% BSA by gentle stirring.
6. Centrifuge the cell suspension at 50 × *g* for 1 min.
7. Wash the pellet, containing parenchymal cells (PC), with Hank's-HEPES buffer twice by centrifuging at 50 × *g* for 1 min.

8. Centrifuge the supernatant, containing non-parenchymal cells (NPC), twice at $50 \times g$ for 1 min.
9. Centrifuge the resultant supernatant twice at $200 \times g$ for 2 min.
10. Resuspend PC and NPC into 2 ml of ice-cold Hank's-HEPES buffer separately.
11. Determine the cell number and cell viability using the Trypan blue exclusion method.
12. Continue with step 5 of Sect. 3.4.

3.6 Comments

DOTMA/Chol (1:1) liposome was prepared and mixed with 50 μg pCMV-Luc to form lipoplexes (see Sects. 3.2 and 3.3). It has been reported that mixing complexes at low ionic strength prevents aggregation, although large complexes resulting from aggregation showed high transfection efficiency in vitro in the case of DNA/transferrin-PEI complexes (Ogris et al. 1998). Based on this report, the complexes in this study were prepared with 5% dextrose solution. After administering the lipoplexes by intravenous injection of the tail vein, the in vivo transfection ability of DOTMA/Chol liposomes was evaluated (See Sect. 3.4) (Kawakami et al. 2000a). The results showed that DOTMA/Chol liposome had high transfection ability in the lungs (Fig. 1). Similar results were reported with 1,2-bis(oleoyloxy)-3-(trimethylammonio) propane (DOTAP)/protamine/DNA complexes (Li and Huang 1997). The authors of that study named this phenomenon "first-passage effect" because the lung is the first capillary bed that the lipoplexes encounter after intravenous injection.

In our laboratory, novel galactosylated and mannosylated cholesterol derivatives (Gal-C4-Chol and Man-C4-Chol) were synthesized (Fig. 2; see Sect. 3.1) and two liposome formulations containing these sugar-modified cholesterol derivatives, DOTMA/Chol/Gal-C4-Chol (1:0.5:0.5) for hepatocyte targeting and Man-C4-Chol/DOPE (6:4) for macrophage targeting, were prepared for receptor-mediated

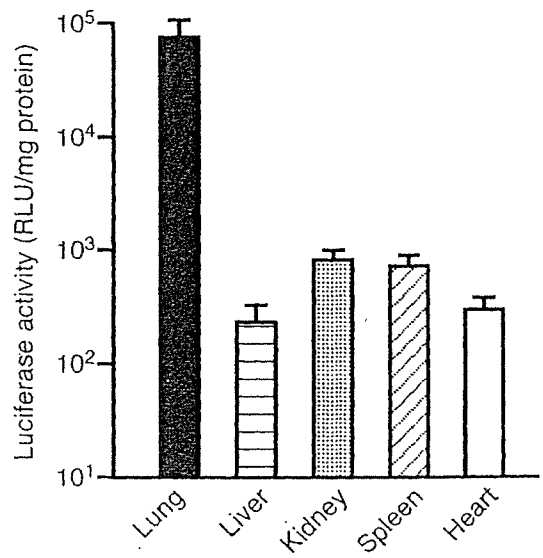

FIG. 1. Transfection activity of DOTMA/Chol (1:1) liposome/DNA complexes after intravenous injection in mice. Plasmid DNA (pCMV-Luc, 30 μg) was complexed with cationic DOTMA/Chol (1:1) liposome at a charge ratio of 2.3. Luciferase activity was measured 6 h post-injection in each organ. Each value represents the mean ± S.D. ($n = 3$)

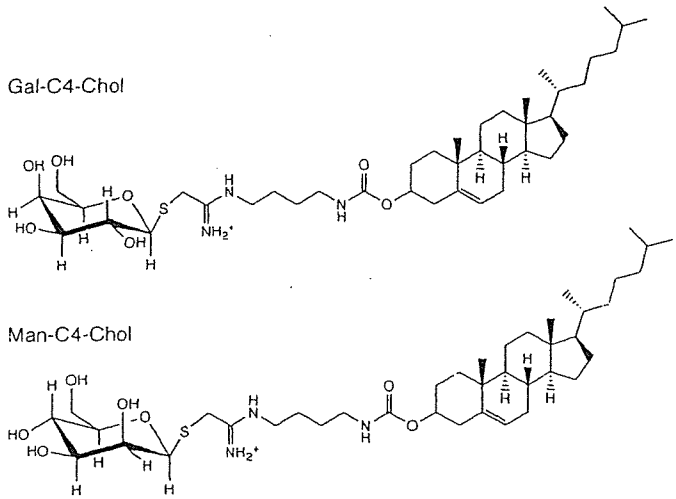

FIG. 2. Structures of novel galactosylated and mannosylated cholesterol derivatives, cholesten-5-yloxy-*N*-(4-((1-imino-2-β-D-thiogalactosyl-ethyl)amino)butyl) formamide (Gal-C4-chol) and cholesten-5-yloxy-*N*-(4-((1-imino-2-β-D-thiomannosyl-ethyl)amino)butyl) formamide (Man-C4-Chol)

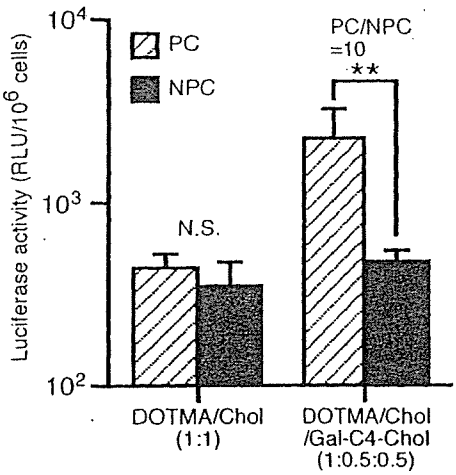

FIG. 3. Intrahepatic gene expression of liposome/DNA complexes after intraportal injection in mice. Plasmid DNA (pCMV-Luc, 50 μg) was complexed with DOTMA/Chol (1:1) and DOTMA/Chol/Gal-C4-chol (1:0.5:0.5) at a charge ratio of 2.3. Luciferase activity was measured 6 h post-injection in parenchymal cells (PC) and non-parenchymal cells (NPC). Statistical analysis was performed by analysis of variance (**$P < 0.01$). *N.S.* Not significant. Each value represents the mean ± S.D. ($n = 3$)

gene delivery to the liver. After formation of the DNA complexes as DOTMA/Chol/Gal-C4-Chol and Man-C4-Chol/DOPE liposomes, these liposome formulations were administrated into mice by intraportal and intravenous injection, respectively. Six hours later, PC and NPC were separated from the liver (see Sect. 3.5), and the hepatic cellular localization of luciferase activity was subsequently evaluated (Kawakami et al. 2000a, b). In the case of DOTMA/Chol/Gal-C4-Chol liposomes, a 10-fold higher luciferase activity was observed in PC than in NPC, while the control liposome, DOTMA/Chol (1:1), showed no significant difference in luciferase activity between PC and NPC (Fig. 3). By contrast, injection of Man-C4-Chol/DOPE liposome yielded significantly higher transgene expression in NPC than in PC, whereas gene expression using the control liposomes, DC-Chol/DOPE (6:4), was slightly higher in NPC (Fig. 4). In addition, galactosylated and mannosylated BSA effectively inhibited gene expression from, respectively, DOTMA/Chol/Gal-C4-Chol and Man-C4-Chol/DOPE liposomes. These results suggesting that hepatic cellular targeting can be achieved by incorporating Gal-C4-Chol or Man-C4-Chol into liposomes at the appropriate ratio of lipids.

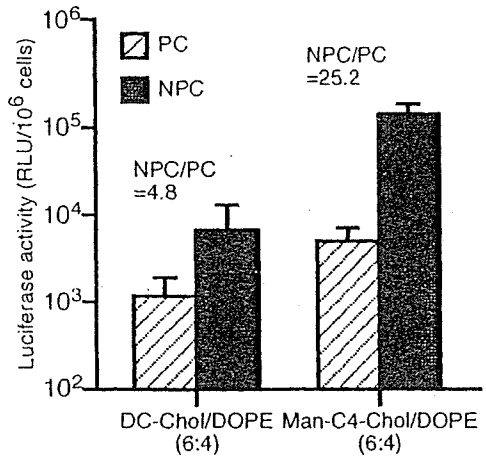

FIG. 4. Intrahepatic gene expression of liposome/DNA complexes after intravenous injection in mice. Plasmid DNA (pCMV-Luc, 50 μg) was complexed with DC-Chol/DOPE (6:4) and Man-C4-chol/DOPE (6:4) at a ratio of 1:7.0 (μg/nmol). Luciferase activity was measured 6 h post-injection in PC and NPC. Each value represents the mean ± S.D. ($n = 3$)

In summary, the luciferase reporter gene assay described in this chapter is a simple and convenient method for monitoring in vivo transgene expression. This system can also be used to evaluate the in vivo transfection ability of other types of gene carrier candidates as well as transgene expression in tumor-bearing animal models.

References

Dash PR, Read ML, Barrett LB, Wolfert MA, Seymour LW (1999) Factors affecting blood clearance and in vivo distribution of polyelectrolyte complexes for gene delivery. Gene Ther 6:643–650

Dash PR, Read ML, Fisher KD, Howard KA, Wolfert M, Oupicky D, Subr V, Strohalm J, Ulbrich K, Seymour LW (2000) Decreased binding to proteins and cells of polymeric gene delivery vectors surface modified with a multivalent hydrophilic polymer and retargeting through attachment of transferrin. J Biol Chem 275:3793–3802

Gao X, Huang L (1991) A novel cationic liposome reagent for efficient transfection of mammalian cells. Biochem Biophys Res Commun 179:280–285

Kawakami S, Yamashita F, Nishikawa M, Takakura Y, Hashida M (1998) Asialoglycoprotein receptor-mediated gene transfer using novel galactosylated cationic liposomes. Biochem Biophys Res Commun 252:78–83

Kawakami S, Fumoto S, Nishikawa M, Yamashita F, Hashida M (2000a) In vivo gene delivery to the liver using novel galactosylated cationic liposome. Pharm Res 17:306–313

Kawakami S, Sato A, Nishikawa M, Yamashita F, Hashida M (2000b) Mannose receptor-mediated gene transfer into macrophages using novel mannosylated cationic liposomes. Gene Ther 7:292–299

Kawakami S, Wong J, Sato A, Hattori Y, Yamashita F, Hashida M (2000c) Biodistribution characteristics of mannosylated, fucosylated, and galactosylated liposomes in mice. Biochim Biophys Acta 1524:258–265

Lee YC, Stowell CP, Krantz MJ (1976) 2-Imino-2-methoxyethyl 1-thioglycosides: new reagents for attaching sugars to proteins. Biochemistry 15:3956–3963

Li S, Huang L (1997) In vivo gene transfer via intravenous administration of cationic lipid-protamine-DNA (LPD) complexes. Gene Ther 4:891–900

Ogris M, Steinlein P, Kursa M, Mechtler K, Kircheis R, Wagner E (1998) The size of DNA/transferring-PEI complexes is an important factor for gene expression in cultured cells. Gene Ther 5:1425–1433

Tandia BM, Vandenbranden M, Wattiez R, Lakhdar Z, Ruysschaert JM, Elouahabi A (2003) Identification of human plasma proteins that bind to cationic lipid/DNA complex and analysis of their sffects on transfection efficiency: implications for intravenous gene transfer. Mol Ther 8:264–273

Templeton NS, Lasic DD, Frederik PM, Strey HH, Roberts DD, Pavlakis GN (1997) Improved DNA:liposome complexes for increased systemic delivery and gene expression. Nature Biotechnol 15:647–652

7
Liposomal *In vivo* Gene Delivery

Shigeru Kawakami, Fumiyoshi Yamashita, and Mitsuru Hashida

Abstract

The goals of a gene delivery system are many-fold, and include: 1) controlled transfection efficacy; 2) controlled cell-specificity of transfected cells; and 3) controlled duration of transgene expression after intravenous administration (Fig. 7.1). Recently, not only plasmid DNA (pDNA) but also oligonucleotides (i.e., siRNA) have become therapeutic candidates. In any case, however, gene therapies have some significant problems because they are polyanionic DNA molecules. These gene therapies are not effective after systemic administration of naked pDNA or oligonucleotide, because of the latters' susceptibility to degradation by nucleases and/or low membrane permeability [1–3]. During the early 1990s, it was shown that sustained and efficient gene transfection could be achieved after local administration of naked pDNA [4]. To date, several methods involving local administration (i.e., intramuscular and intratumoral) of naked pDNA have been studied for the application of gene therapy [5–8]. Moreover, electroporation – the application of a controlled electric field to facilitate cell permeabilization – has been shown to enhance the transfection activity of administered pDNA [9, 10]. However, compared with these local applications of naked pDNA, systemic application by vascular routes is able to transfect the gene to a large number of cells throughout the entire tissue. Thus, an *in vivo* gene delivery system via the intravascular route should be developed. Various transfection characteristics are important when highly efficient gene therapy is required to treat a variety of refractory diseases (see also Part I, Chapter 7 and Part VI, Chapters 1, 3, and 6). Non-viral vectors should circumvent some of the problems occurring with viral vectors, such as endogenous virus recombination, oncogenic effects, and unexpected side effects [11, 12]. These non-viral vectors can be divided into two general groups: cationic liposomes, and polymers. Among various types of non-viral vectors, cationic liposome-mediated gene transfection is one of the most promising approaches due to the high transfection efficiency, notably in the lung after intravenous administration (see Table 7.1). Moreover, recent advances in gene delivery technologies now enable us to deliver the pDNA into liver, heart (see Part I, Chapter 6), macrophages, and cancer cells via uptake by cell-specific receptors (Table 7.1). This chapter focuses on the progress of research into cationic liposome-mediated *in vivo* gene transfer.

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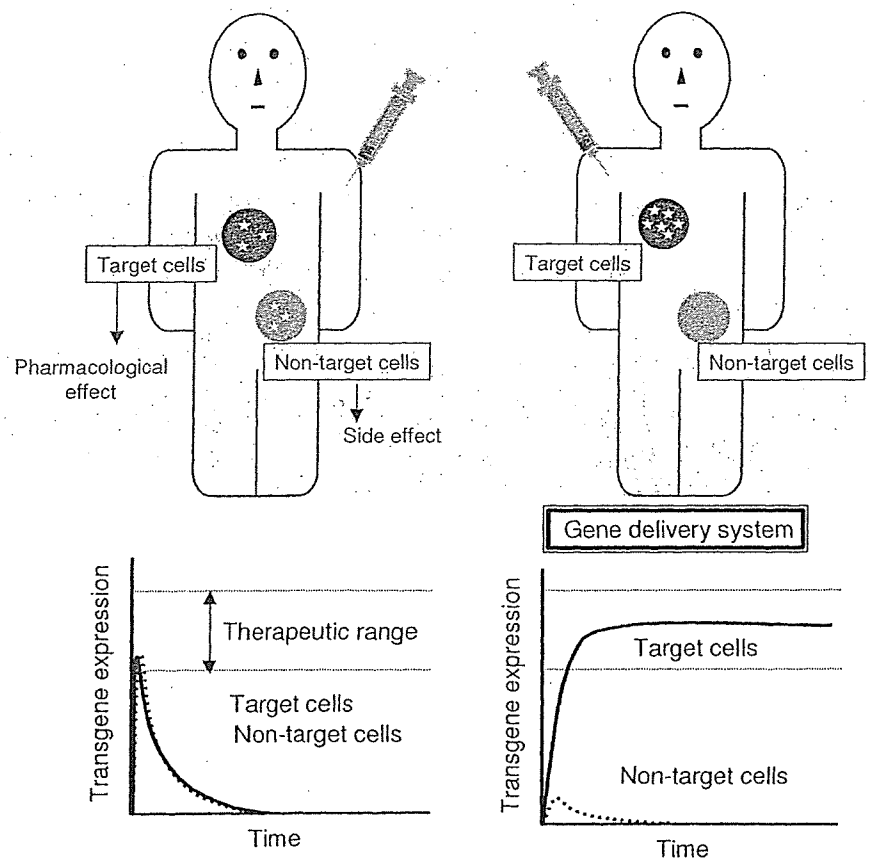

Fig. 7.1 The goal of a gene delivery system. Upper panel: After intravenous administration, transgene expression (star) should occur in target cells only, and not in non-target cells. Lower panel: The levels of transgene expression should be in the therapeutic levels at moderate terms.

Table 7.1 Liposomal *in vivo* gene transfer approaches

Target (receptor)	Gene	Carrier (lipid composition)	Reference
Lung	p-CAT	DOTMA/DOPE	14
	p-CAT	DOTMA/DOPE (Lipofectin®), DDAB/DOPE (Lipofect ACE®)	15
	p-CAT	DOTIM/Chol	16
	p-CAT	GAP-DLRIE/DOPE	17
	p-Luc	DOTAP/Chol-protamine (LPD)	18
	p-CAT	DOTIM/Chol	19
	p-Luc	DOTMA/Chol, DOTAP/Chol, DOTMA, DOTAP	20
	p-Luc	DOTMA/Tween 80	21
	p-CAT	DOTMA, DOLCE, MMCE, EDOPC, EPMPC	23
	p-Luc	DOTMA/Chol	25
	p-IL-2 or 12	DOTAP/Chol	27
	p-mIFNβ	DOTMA/Chol	28

Table 7.1 (continued)

Target (receptor)	Gene	Carrier (lipid composition)	Reference
Hepatocytes (asialoglycoprotein receptor)			
	p-CAT	Palmitoyl asialofetuin/PC/TMAG with EDTA	42
	p-Luc	Gal-C4-Chol/DOTMA/Chol	45
	p-Luc	Gal-C4-Chol/DOTMA/Chol	45
Macrophages (mannose receptor)			
	p-Luc	Man-C4-Chol/DOPE	50
	p-Luc	Man-C4-Chol/DOTMA/Chol	51
	p-Luc	Man-C4-Chol/DOPE	52
	p-Luc	Man-C4-Chol/DOPE	53
	p-OVA	Man-C4-Chol/DOPE	62
Tumor (folate receptor)			
	p-Luc	Folate-PEG-Chol/DOPE/RPR209120	64
	p-Luc	Folate-PEG-Chol/DOPE/RPR209120	68
	Antisense DNA	Folate-PEG-DSPE/Chol/eggPC	69
Brain (transferrin receptor)			
	p-βGal	Transferrin mAb-PEG-DSPE /DDAB/POPC	78
	p-βGal	Transferrin/Insulin mAb-PEG-DSPE /DDAB/POPC	79
	p-siRNA(EGFR)	Transferrin/Insulin mAb-PEG-DSPE /DDAB/POPC	80

Abbreviations

APC	antigen-presenting cell
BSA	bovine serum albumin
CAT	chloramphenicol acetyltransferase
CMV	cytomegalovirus
DC	dendritic cell
DDAB	didodecyldimethylammonium bromide
DEAE	dimethylaminoethyl
DOLCE	oleyl oleoyl L-carnitine ester
DOPE	dioleoylphosphatidylethanolamine
DOTAP	1,2-bis (oleyloxy)-3-(triethylammonium)propane
DOTMA	*N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride
EDOPC	ethyl dioleoyl phosphatidylcholine
EDTA	ethylen-diamin-tetra-acid
EGFR	epithelial growth factor receptor
ELISA	enzyme-linked immunosorbent assay
EPMPC	ethyl palmitoyl myristyl phosphatidylcholine
βGal	β-galactosidase
Gal-C4-Chol	Cholesten-5-yloxy-*N*-(4-((1-imino-2-D-thiogalactosylethyl)amino)alkyl)formamide
GAP-DLRIE	(±)-*N*-(3-aminopropyl)-*N*,*N*-dimethyl-2,3-bis(dodecyloxy)-1-propanaminium bromide
GFAP	glial fibrillary acidic protein

hEGFR	human epithelial growth factor receptor
HIR	human insulin receptor
HIV	human immunodeficieny virus
ICR	Crlj:CD1
IFN	interferon
IL	interleukin
KB	cell name
Luc	luciferase
mAb	monoclonal antibody
Man-C4-Chol	cholesten-5-yloxy-*N*-(4-((1-imino-2-D-thiomannosyl-ethyl)amino)alkyl)formamide
MMCE	myristyl myristoyl carnitine ester
NK	natural killer cells
NPC	non-parenchymal cells
OVA	ovalbumine
OX	antibody name
PC	parenchymal cells
pDNA	plasmid DNA
PEG	polyethyleneglycol
POPC	1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphocholine
RES	reticuloendothelial system
RME	receptor-mediated endocytosis
siRNA	small interfering RNA
TMAG	*N*-(α-trimethylammonium-acetyl)-didodecyl-D-glutamate chloride
TNF-α	tumor necrosis factor-alpha

7.1 Cationic Charge-mediated *In vivo* Gene Transfer to the Lung

Cationic liposome-mediated *in vitro* gene transfection was first reported by Felgner et al. in 1987 [13]. In this approach, pDNA is mixed with preformed small cationic vehicles to form pDNA/lipid complexes based on electrostatic interaction; these complexes can then interact with target cells and be taken up by them. During the late 1990s, several studies showed that intravenous administration of pDNA/cationic liposome complexes (i.e., lipoplex) leads to systemic gene expression. After intrave-

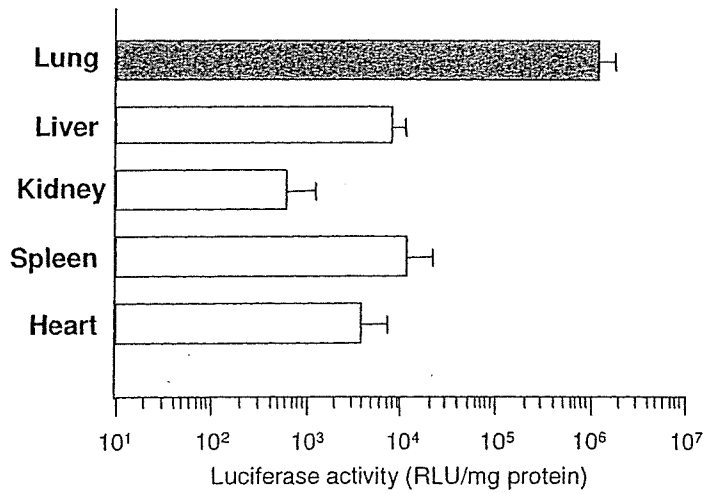

Fig. 7.2 Transgene expression in various tissues after intravenous administration of pCMV-luciferase (Luc) (30 μg) complexed with cationic liposomes (DOTMA/Chol liposomes) in female ICR mice. Lipoplex were prepared at a charge ratio (−:+) of 1.0:3.1 in 5% dextrose. Each value represents the mean ± SD of at least three experiments.

nous administration of lipoplex, the lung shows the highest amount of gene expression among the major organs, and the lung endothelial cells are the main contributor to transgene expression [14–23]. We and others have confirmed that the gene expression level in the lung is 100- to 10 000-fold higher than that in the liver and spleen (Fig. 7.2).

In order to establish a strategy for efficient, safe non-viral gene delivery systems, a thorough understanding of the *in vivo* disposition characteristics of the lipoplex is necessary. As for the distribution of the lipoplex, Mahato et al. have described the disposition characteristics of [^{32}P] pDNA/cationic liposome complexes after intravenous injection in mice [24]. Rapid clearance of lipoplex from the circulation was observed, followed by extensive accumulation in the lungs and liver. With regard to the type of liver cells involved, the lipoplex was predominantly taken up by non-parenchymal cells after intravenous injection. A high degree accumulation of lipoplex would explain the high gene expression level in the lung. Some studies have suggested that the lipoplex aggregates in the blood compartments due to its strong positive charge and becomes entrapped in the lung capillaries; thus, interaction with erythrocytes could be an important factor for the lung accumulation of lipoplex [25, 26].

Taking these factors into consideration, the intravenous administration of lipoplex may represent a promising gene delivery method to treat pulmonary diseases. Simple gene delivery can be an effective approach for the treatment of lung diseases, including pulmonary metastasis of tumor cells. Indeed, the intravenous administration of interferon (IFN)-β- and interleukin (IL)-12-encoding pDNA has been shown to provide effective treatment in a murine pulmonary cancer metastasis model [27, 28].

However, it has been also reported that proinflammatory cytokines trigger damage and apoptosis of vascular endothelial cells after intravenous administration (of lipoplex) [29]. Large amounts of proinflammatory cytokines are produced in the blood after intravenous injection, since bacterially derived pDNA is recognized as foreign material by vertebrate cells [30, 31]. Unmethylated CpG sequences in pDNA, occurring at a higher frequency in bacterial DNA, have been reported to have strong stimulatory effects on lymphocytes, natural killer (NK) cells, dendritic cells, and macrophages to induce production of large quantities of proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, IFN-γ, and IL-12 [32, 33]. Li et al. [29] reported that, after intravenous administration of lipoplex, these proinflammatory cytokines are not only toxic to experimental animals but also cause gene inactivation, including a short duration of gene expression and resistance to repeated dosing at frequent intervals. Freidmark et al. [34] reported that intratracheal administration of lipoplex to the lungs induces the production of proinflammatory cytokines [34]. We have shown previously that pretreatment with gadolinium chloride ($GdCl_3$), which is known transiently to deplete liver Kupffer cells and spleen macrophages [35], dramatically reduces the serum levels of these proinflammatory cytokines and the liver accumulation of lipoplex, suggesting that tissue macrophages involving liver Kupffer cells and spleen macrophages are closely involved in proinflammatory cytokine production following intravenous administration of lipoplex [36]. Whilst these proinflammatory cytokine responses represent an important factor in the development of safe and effective non-viral gene therapies to treat pulmonary diseases, little detailed information is available and therefore

further research is warranted before clinical application is possible.

7.2 Asialoglycoprotein Receptor-mediated *In vivo* Gene Transfer to Hepatocytes

For cell-specific delivery, the 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 using this the RME approach [37, 38]. Hepatocytes exclusively express large numbers of high-affinity cell-surface receptors that can bind asialoglycoproteins and subsequently internalize them to the cell interior.

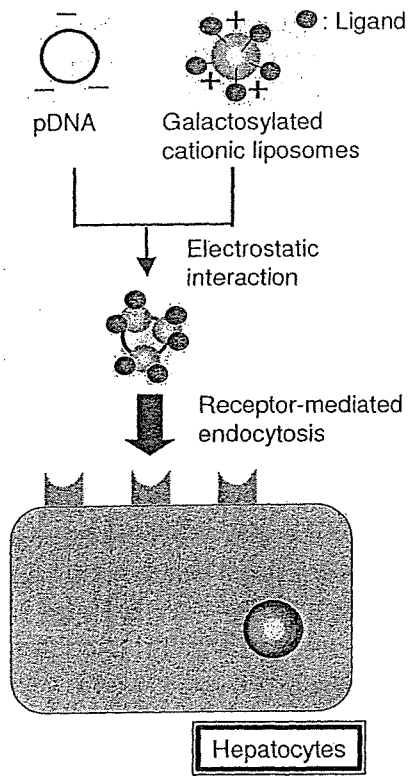

Fig. 7.3 Scheme for hepatocyte-selective gene delivery by complexation with galactosylated carrier via asialoglycoprotein receptor-mediated endocytosis.

During the late 1980s, Wu et al. demonstrated successful *in vivo* gene transfer to the liver using poly-L-lysine linked with asialoorosomucoid [39, 40]. Successful *in vivo* gene expression after intravenous injection has been also reported for glycosylated poly-L-lysine [41]. However, its transfection efficacy appears to be low and, therefore, this needs to be improved for gene therapy. In general, the transfection efficacy of cationic liposome is higher than that of cationic polymers; therefore, this mechanism would be an effective way of achieving hepatocyte targeting using galactosylated cationic liposomes. The galactosylation of liposomes can be achieved by coating with either glycoproteins or galactose-conjugated synthetic lipids (Fig. 7.3).

With regard to targeted gene delivery by liposomes, Hara et al. [42] reported that asialofetuin-labeled liposomes encapsulating pDNA were taken up by asialoglycoprotein RME using cultured hepatocytes, and showed the highest hepatic gene expression to be achieved after intraportal injection with a preload of EDTA [42]. The introduction of asialoglycoproteins to liposomes is complicated however, and several problems are associated with the carriers, including reproducibility and immunogenicity. The *in vivo* application of EDTA is also limited. Thus, the low molecular-weight glycolipids appear to be more promising due to their low immunogenicity and high reproducibility. Remy et al. [43] reported the feasibility of using galactose-presenting lipopolyamine vectors for targeted gene transfer into hepatoma cells under *in vitro* conditions. The inclusion of galactose residues in the electrically neutral complex increased transgene expression which approached the level obtained with a large excess of cationic liposomes alone. For *in vivo* hepatocyte-selective

gene transfection using by low molecular-weight glycolipids, we designed a novel galactosylated cationic cholesterol derivative, cholesten-5-yloxy-*N*-(4-((1-imino-2-D-thiogalactosylethyl)amino)alkyl)formamide (Gal-C4-Chol), for preparation of the galactosylated lipoplex [44–46]. Gal-C4-Chol is a specially designed galactosylated lipid for the modification of cationic lipids because the galactose moieties are stably fixed to the liposomal membrane under *in vivo* conditions.

In fact, 75% of a dose of Gal-C4-Chol-containing liposome/[^{32}P] pDNA complex was found in the liver at only 1 minute after intraportal administration [45]. The hepatic gene expression of pDNA complexed with Gal-C4-Chol liposomes was more than 10-fold greater than that of pDNA complexed with conventional cationic liposomes. When gene expression was examined by determining intrahepatic cellular levels, that of liver parenchymal cells (PC) of pDNA complexed with Gal-C4-Chol-containing liposomes was significantly higher than that of liver non-parenchymal cells (NPC). In contrast, there was little difference in the gene expression of PC and NPC of conventional cationic liposomes. In addition, when an excess of galactosylated bovine serum albumin (BSA) was injected intravenously 5 minutes before injection of galactosylated lipoplex, gene expression in the liver was significantly reduced, which suggested that uptake occurred via asialoglycoprotein RME.

The level of *in vivo* gene expression by galactosylated lipoplex was less than would be expected based on *in vitro* results, however. Hence, several barriers must be 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 disposition profiles of galactosylated lipoplex using rat liver perfusion techniques [47]. This allowed us to determine the uptake characteristics of various substances under different experimental conditions, with the structure of the liver remaining intact. In these studies, penetration of the galactosylated lipoplex through the hepatic fenestrated endothelium to the parenchymal cells was shown to be greatly restricted in perfused rat liver, in spite of the small size of the galactosylated lipoplex (ca. 120 nm), with regard to crossing the fenestrae. It has been reported that, following intravenous administration, the lipoplex interacts with erythrocytes [25, 26], which suggests that the galactosylated lipoplex aggregates by non-specific interaction with erythrocytes. In order to enhance the transfection activity in hepatocytes, these non-specific interactions between blood components and galactosylated lipoplex must be controlled.

7.3 Mannose Receptor-mediated *In vivo* Gene Transfer to Macrophages

Macrophages are important targets for the gene therapy of a number of diseases, including Gaucher's disease and human immunodeficiency virus (HIV) infection, although the process of gene transfection in such cases is not easy (see Part II, Chapter 7). The use of non-viral 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. The use of DEAE-dextran represents one approach for gene delivery to macrophages *in vitro* [48], but the method is generally not suitable for *in vivo* use due to problems associated with cellular toxicity, low efficiency, or non-specific biodistribution. Erbacher et al. [49] investigated the suitability of various glycosylated poly(L-ly-

sine) derivatives for the introduction of pDNA into human monocyte-derived macrophages, and found that mannosylated poly(L-lysine) exhibited high transfection activity. However, these authors 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 RME; 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 a novel mannosylated cholesterol derivative, cholesten-5-yloxy-*N*-(4-((1-imino-2-D-thiomannosylethyl) amino)-alkyl)formamide (Man-C4-Chol), for mannose receptor-mediated gene transfection to macrophages [50–53], which are known to express large numbers of mannose receptors on their surface. In primary cultured mouse peritoneal macrophages, pDNA, when complexed with Man-C4-Chol liposomes, showed higher transfection activity than that complexed with conventional cationic liposomes [50]. The presence of 20 mM mannose significantly inhibited the transfection efficiency of pDNA complexed with Man-C4-Chol liposomes, suggesting that the mannosylated lipoplex is recognized and taken up by the mannose receptors on macrophages.

After intravenous administration, the highest gene expression was observed in the liver after intravenous injection of mannosylated lipoplex in mice (Fig. 7.4). In addition, gene expression with mannosylated lipoplex in the liver was observed preferentially in the liver NPC, and was significantly reduced by predosing with mannosylated BSA. These results suggest that mannosylated lipoplex exhibits high transfection activity in NPC due to recognition by mannose receptors. Unlike the case of the Gal-C4-Chol-containing liposome/pDNA complex, cell-selective gene transfection can be achieved by the intra-

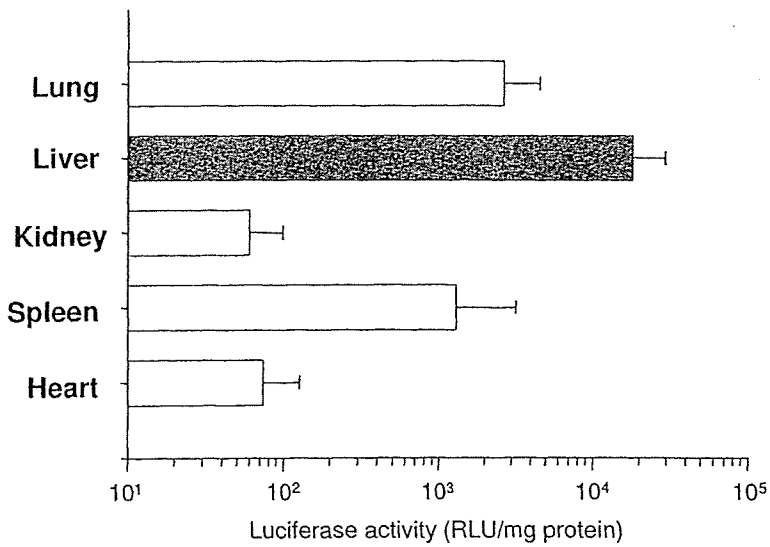

Fig. 7.4 Transgene expression in various tissues after intravenous administration of pCMV-Luc (50 μg) complexed with mannosylated liposomes (Man-C4-Chol/DOPE liposomes) in female ICR mice. Mannosylated lipoplex were prepared at a charge ratio (−:+) of 1.0:2.3 in 5% dextrose. Each value represents the mean ± SD of at least three experiments.

venous administration of the Man-C4-Chol-containing liposome/pDNA complex [52]. This phenomenon could be explained by the fact that, in the liver and spleen, macrophages are present around endothelial cells; they are in contact with – but do not pass through – the sinusoids (100–200 nm). Hence, mannosylated lipoplex is effective in an NPC-selective gene transfection system, even when administered intravenously.

DNA vaccination – the administration of DNA-encoding antigen gene 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 [54–56] (see also Part I, Chapter 7 and Part VI, Chapter 3). This cell-mediated immune response plays a crucial role in the immune response against cancer and infectious diseases [57]. To date, many attempts have been made to use naked pDNA-based immunization, administered either intramuscularly or subcutaneously, to produce humoral and cellular immunity and to demonstrate its potency in non-human primates. Immunological studies have shown that gene transfection and subsequent activation of dendritic cells are key events in the development of immunity following DNA vaccination [58]. However, recent clinical trials have shown that the immune response following topical injection of naked pDNA solution is insufficient [59, 60], indicating that further improvements in the transfection efficiency involving some pharmaceutical modification are needed for DNA vaccine therapy.

Perrie et al. [61] reported that cationic liposomes enhance gene expression in draining lymph nodes, which contain many antigen-presenting cell (APC) populations, after intramuscular administration, as well as increasing the antigen-specific antibody response [61]. Although the adjuvant effect of cationic liposomes is widely accepted, the lack of cell-specificity of cationic liposomes after *in vivo* application is regarded as limiting their transfection efficiency to APCs and the resulting adjuvant efficiency. For efficient gene therapy, non-viral vectors offer the major advantages of sustained effect, high-level transgene expression with minimal toxicity, and few immunological side effects. It is well known that APCs express high levels of mannose receptors, which have a high capacity for the uptake of antigens that have mannose residues. Therefore, one promising approach for efficient gene delivery to APCs is the attachment of mannose residues to cationic liposomes for cell-selective gene transfection. The mannosylated liposome formulation allows the development of a DNA vaccine with suitable pharmaceutical properties for APC targeting under *in vivo* conditions; therefore, this carrier system is expected to improve the immune response of this novel DNA vaccine. Recently, we showed that the targeted delivery of DNA vaccine by Man-C4-Chol liposomes is a potent method of DNA vaccine therapy [62] (Table 7.2). Although further improvements in transfection efficacy are required, targeted delivery of DNA vaccine to dendritic cells (DCs) could improve future *in vivo* DNA vaccine therapies.

7.4 Folate Receptor-mediated *In vivo* Gene Transfer to Cancer Cells

In recent years, over 50% of all clinical gene therapy trials, including immune gene therapy and gene-directed enzyme

Table 7.2 Antigen (OVA)-specific proliferation response of spleen cells and IFN-γ secretion from mice immunized with naked pCMV-OVA and that complexed with DC-Chol or Man-C4-Chol liposomes.

Group	Proliferation index [OVA(+)/OVA (–)]	IFN-γ release [pg mL^{-1}]	
		OVA(–)	OVA(+)
No treatment	1.183	343.3	130.0
Naked pCMV-OVA	1.208	248.2	904.1
DC-Chol/DOPE complex	1.565	503.9	2430.7
Man-C4-Chol/DOPE complex	1.733	879.2	3795.7

After immunization by intravenous administration, spleen cells were cultured and stimulated with 100 μg OVA. Proliferation of spleen cells was evaluated by AlamarBlue™. IFN-γ in the culture medium was evaluated by ELISA. Each value represents the mean of five assays using a single pool of spleen cell suspension from five mice in each group.

prodrug therapy, have been targeted at the treatment of cancer [63]. The most important factor for gene therapy is an efficient *in vivo* gene transfection methodology. Folate receptors are known to be overexpressed in a large fraction of human tumors, but they are only minimally distributed in normal tissues; therefore, folate receptors have also been used as tumor-targeting ligands for several drug delivery systems. To achieve tumor-selective gene delivery, Hofland et al. synthesized folate-polyethyleneglycol (PEG)-lipid derivatives to prepare folate-modified cationic liposomes [64]. PEG is introduced into the spacer part of folate-modified lipids to avoid reticuloendothelial system (RES) uptake and prolong the circulation time of conventional liposomes [65–67]. After intravenous administration of folate-PEG lipid-liposome complexed with pDNA, lung accumulation and gene expression was reduced in proportion to the quantity of lipid used. In contrast, gene expression in tumor tissue remained constant in liposomes containing up to 3 mol% of folate-PEG lipid. Even at 3 mol% of folate-PEG lipid-containing liposomes complex, gene expression levels in the lung and tumor were almost identical; thus, further improvements are required for tumor-selective gene delivery after intravenous administration.

After intraperitoneal injection into a murine disseminated peritoneal tumor model, however, folate lipoplex formulations produced an approximately 10-fold increase in tumor-associated gene expression, compared with conventional complex [68]. When gene expression was measured in tumors and various peritoneal organs after intraperitoneal administration, the highest gene expression was observed in tumor cell ascites, followed by solid tumors. Thus, intraperitoneal administration of the folate-PEG-lipid-containing liposome complex may be more suitable for peritoneal dissemination.

More recently, the same group attempted to deliver an antisense oligonucleotide complexed with folate liposomes in a series of *in vitro* and *in vivo* experiments [69]. Although oligonucleotide-loaded folate liposomes were effectively associated with KB cells

(which express folate receptors on their cell surface), no improvement was observed after intravenous administration to mice inoculated with KB cells. These authors concluded that folate liposomes can effectively deliver oligonucleotides into folate receptor-bearing cells *in vitro*, but additional barriers exist *in vivo* that prevent or reduce effective tumor uptake and retention.

7.5 Transferrin Receptor-mediated *In vivo* Gene Transfer to Brain

The transferrins are a structurally related class of metal-binding glycoproteins of approximately 80 kDa in size, the primary function of which is the binding and transportation of non-heme iron [70–73]. High levels of transferrin receptor expression have been demonstrated in the brain capillary endothelium [74], cancer cells [75–77], liver, and spleen [78]. Recently, Pardridge et al. [78] described brain-specific gene delivery systems using monoclonal antibody (mAb)-modified pegylated liposomes via transferrin receptors on the brain capillary endothelium. First, the murine OX 26 mAb to the rat transferrin receptor was used to target the pegylated immunoliposome carrying the pSV-β-galactosidase plasmid to tissues *in vivo* [78]. In addition, gene expression of the exogenous gene in brain, liver, and spleen was demonstrated by β-galactosidase histochemistry, which showed persistent gene expression for at least 6 days after intravenous administration. The persistent gene expression was confirmed by Southern blot analysis. In order to obtain brain-specific gene expression, these authors selected the human glial fibrillary acidic protein (GFAP) promoter encoding pDNA [79]. After intravenous administration of GFAP/β-galactosidase pDNA immunoliposomes, the exogenous gene was expressed in the brain. In contrast, there was no expression of the transgene in mouse spleen, liver, heart, and lung. These results indicated that brain-specific gene expression is possible after intravenous administration of transferrin receptor mAb-modified pegylated liposomes encapsulated in GFAP promoter encoding pDNA.

More recently, it was shown that, following intravenous administration, transferrin receptor mAb-modified pegylated liposomes/siRNA encoding pDNA complex could block the human epithelial growth factor receptor (hEGFR) expression by RNA interference [80]. In this new targeting system, two different receptors are targeted: one is the rat 8D3 mAb to the mouse transferrin receptor, which enables transport of the lipoplex across the mouse blood–brain barrier forming the microvasculature of intracranial cancer; the other mAb targets the human insulin receptor (HIR) that is expressed on the plasma membrane of human brain cancer. A short hairpin RNA is directed at a specific sequence in the human EGFR mRNA, and this siRNA encoding pDNA can be encapsulated in these double mAb-modified immunoliposomes. After weekly intravenous administration to mice with intracranial human brain cancer, RNA interference gene therapy reduced the tumor expression of immunoreactive EGFR, resulting in an 88% increase in survival time of mice with advanced intracranial brain cancer.

7.6 Conclusions

Successful *in vivo* gene therapy requires the development of a rational gene delivery technology that satisfies various require-

ments for each target disease. The development of cell-specific, non-viral gene delivery carriers is required to achieve effective *in vivo* gene therapy. Further basic and clinical studies should allow successful *in vivo* gene therapy in the near future, and pave the way to successful modern biopharmaceuticals.

References

1 K. Kawabata, Y. Takakura, M. Hashida, *Pharm. Res.* 1995, 12, 825–830.
2 D. Lew, S.E. Parker, T. Latimer, et al., *Hum. Gene Ther.* 1995, 6, 553–564.
3 R.I. Mahato, S. Takemura, K. Akamatsu, et al., *Biochem. Pharmacol.* 1997, 53, 887–895.
4 J.A. Wolff, R.W. Malone, P. Williams, et al., *Science* 1990, 247, 1465–1468.
5 T. Nomura, S. Nakajima, K. Kawabata, et al., *Cancer Res.* 1997, 57, 2681–2686.
6 K. Hiraoka, H. Koike, S. Yamamoto, et al., *Circulation* 2003, 108, 2689–2696.
7 T. Nomura, K. Yasuda, T. Yamada, et al., *Gene Ther.* 1999, 6, 121–129.
8 K.W. Liang, M. Nishikawa, F. Liu, et al., *Gene Ther.* 2004, 11, 901–908.
9 H. Aihara, J. Miyazaki, *Nat. Biotechnol.* 1998, 16, 867–870.
10 M.L. Lucas, L. Heller, D. Coppola, et al., *Mol. Ther.* 2002, 5, 668–675.
11 M. Hashida, M. Nishikawa, F. Yamashita, et al., *Adv. Drug Deliv. Rev.* 2001, 52, 187–196.
12 T. Niidome, L. Huang, *Gene Ther.* 2002, 9, 1647–1652.
13 P.L. Felgner, T.R. Gadek, M. Holm, et al., *Proc. Natl. Acad. Sci. USA* 1987, 84, 7413–7417.
14 N. Zhu, D. Liggitt, Y. Liu, et al., *Science* 1993, 261, 209–211.
15 R.I. Mahato, K. Kawabata, Y. Takakura, et al., *J. Drug Target.* 1995, 3, 149–157.
16 Solodin, C.S. Brown, M.S. Bruno, et al., *Biochemistry* 1995, 34, 13537–13544.
17 C.J. Wheeler, P.L. Felgner, Y.J. Tsai, et al., *Proc. Natl. Acad. Sci. USA* 1996, 93, 11454–11459.
18 S. Li, L. Huang, *Gene Ther.* 1997, 4, 891–900.
19 J.W. McLean, E.A. Fox, P. Baluk, et al., *Am. J. Physiol.* 1997, 273, H387–H404.
20 Y.K. Song, F. Liu, S. Chu, et al., *Hum. Gene Ther.* 1997, 8, 1585–1594.
21 F. Liu, H. Qi, L. Huang, et al., *Gene Ther.* 1997, 4, 517–523.
22 L. Huang, S. Li, *Nat. Biotechnol.* 1997, 15, 620–621.
23 R.I. Mahato, K. Anwer, F. Tagliaferri, et al., *Hum. Gene Ther.* 1998, 9, 2083–2099.
24 R.I. Mahato, K. Kawabata, T. Nomura, et al., *J. Pharm. Sci.* 1995, 84, 1267–1271.
25 F. Sakurai, T. Nishioka, H. Saito, et al., *Gene Ther.* 2001, 8, 677–686.
26 H. Eliyahu, N. Servel, A.J. Domb, et al., *Gene Ther.* 2002, 9, 850–858.
27 S.W. Dow, R.E. Elmslie, L.G. Fradkin, et al., *Hum. Gene Ther.* 1999, 10, 2961–2972.
28 F. Sakurai, T. Terada, M. Maruyama, et al., *Cancer Gene Ther.* 2003, 10, 661–668.
29 S. Li, S.-P. Wu, M. Whitmore, et al., *Am. J. Physiol.* 1999, 276, L796–L804.
30 Y. Tan, S. Li, B.R. Bruce, et al., *Hum. Gene Ther.* 1999, 10, 2153–2161.
31 S. Loisel, C.L. Gall, L. Doucet, et al., *Hum. Gene Ther.* 2001, 12, 685–696.
32 M. Krieg, A.K. Yi, S. Matson, et al., *Nature* 1995, 374, 546–549.
33 M. Krieg, T. Wu, R. Weeratna, et al., *Proc. Natl. Acad. Sci. USA* 1998, 95, 12631–12636.
34 D. Freimark, H.P. Blezinger, V.J. Florack, et al., *J. Immunol.* 1998, 160, 4580–4586.
35 M.J. Hardonk, F.W.J. Dijkhuis, C.E. Hulstaert, et al., *J. Leukoc. Biol.* 1992, 52, 296–302.
36 F. Sakurai, T. Terada, K. Yasuda, et al., *Gene Ther.* 2002, 9, 1120–1126.
37 S. Kawakami, F. Yamashita, K. Nishida, et al., *Crit. Rev. Ther. Drug Carrier Syst.* 2002, 19, 171–190.
38 M. Ogris, E. Wagner, *Somat. Cell Mol. Genet.* 2002, 27, 85–95.
39 G.Y. Wu, C.H. Wu, *J. Biol. Chem.* 1988, 263, 14621–14624.
40 N.R. Chowdhury, C.H. Wu, G.Y. Wu, et al., *J. Biol. Chem.* 1993, 268, 11265–11271.
41 J.C. Perales, T. Ferkol, H. Beegen, et al., *Proc. Natl. Acad. Sci. USA* 1994, 91, 4086–4090.
42 T. Hara, Y. Aramaki, S. Takada, et al., *Gene Ther.* 1995, 2, 784–788.
43 J.S. Remy, A. Kichler, V. Mordvinov, et al., *Proc. Natl. Acad. Sci. USA* 1995, 92, 1744–1748.
44 S. Kawakami, F. Yamashita, M. Nishikawa, et al., *Biochem. Biophys. Res. Commun.* 1998, 252, 78–83.

45 S. Kawakami, S. Fumoto, M. Nishikawa, et al., *Pharm. Res.* **2000**, 17, 306–313.

46 S. Fumoto, S. Kawakami, Y. Ito, et al., *Mol. Ther.* 2004, 10, 719–729.

47 S. Fumoto, F. Nakadori, S. Kawakami, et al., *Pharm. Res.* **2003**, 20, 1452–1459.

48 A.P. Rupprecht, D.L. Coleman, *J. Immunol. Methods* **1991**, 144, 157–163.

49 P. Erbacher, M.T. Bousser, J. Raimond, et al., *Hum. Gene Ther.* **1996**, 7, 721–729.

50 S. Kawakami, A. Sato, M. Nishikawa, et al., *Gene Ther.* **2000**, 7, 292–299.

51 S. Kawakami, A. Sato, M. Yamada, et al., *S.T.P. Pharma Sci.* **2001**, 11, 117–120.

52 S. Kawakami, Y. Hattori, Y. Lu, et al., *Pharmazie* **2004**, 59, 405–408.

53 M. Yamada, M. Nishikawa, S. Kawakami, et al., *J. Control. Release* **2004**, 98, 157–167.

54 J.B. Ulmer, J.J. Donnelly, S.E. Parker, et al., *Science* **1993**, 259, 1745–1749.

55 R. Schirmbeck, W. Bohm, K. Ando, et al., *J. Virol.* **1995**, 69, 5929–5934.

56 L. Doolan, M. Sedegah, R.C. Hedstrom, et al., *J. Exp. Med.* **1996**, 183, 1739–1946.

57 T. Nishimura, M. Nakui, M. Sato, et al., *Cancer Chemother. Pharmacol.* **2000**, 46, S52–S61.

58 O. Akbari, N. Panjwani, S. Garcia, et al., *J. Exp. Med.* **1999**, 189, 169–178.

59 M.J. Roy, M.S. Wu, L.J. Barr, et al., *Vaccine* **2000**, 19, 764–778.

60 S.A. Rosenberg, J.C. Yang, R.M. Sherry, et al., *Hum. Gene Ther.* **2003**, 14, 709–714.

61 Y. Perrie, P.M. Frederik, G. Gregoriadis, *Vaccine* **2001**, 19, 3301–3310.

62 Y. Hattori, S. Kawakami, S. Suzuki, et al., *Biochem. Biophys. Res. Commun.* **2004**, 317, 992–999.

63 R. Dass, M.A. Burton, *J. Pharm. Pharmacol.* **1999**, 51, 755–770.

64 H.E. Hofland, C. Masson, S. Iginla, et al., *Mol. Ther.* **2002**, 5, 739–744.

65 L. Klibanov, K. Maruyama, V.P. Torchilin, et al., *FEBS Lett.* **1990**, 268, 235–237.

66 L. Klibanov, K. Maruyama, A.M. Beckerleg, et al., *Biochim. Biophys. Acta* **1991**, 1062, 142–148.

67 T.M. Allen, C. Hansen, F. Martin, et al., *Biochim. Biophys. Acta* **1991**, 1066, 29–36.

68 J.A. Reddy, C. Abburi, H. Hofland, et al., *Gene Ther.* **2002**, 9, 1542–1550.

69 P. Leamon, S.R. Cooper, G.E. Hardee, *Bioconjug. Chem.* **2003**, 14, 738–747.

70 P. Aisen, E.B. Brown, *Semin. Hematol.* **1977**, 14, 31–53.

71 P. Aisen, I. Listowsky, *Annu. Rev. Biochem.* **1980**, 49, 357–393.

72 N. Baker, P.F. Lindley, *J. Inorg. Biochem.* **1992**, 47, 147–160.

73 R. Richardson, P. Ponka, *Biochim. Biophys. Acta* **1997**, 1331, 1–40.

74 N. Shi, W.M. Pardridge, *Proc. Natl. Acad. Sci. USA* **2000**, 97, 7567–7572.

75 M. Kursa, G.F. Walker, V. Roessler, et al., *Bioconjug. Chem.* **2003**, 14, 222–231.

76 J. Hildebrandt, M. Iyer, E. Wagner, et al., *Gene Ther.* **2003**, 10, 758–764.

77 K. Maruyama, O. Ishida, S. Kasaoka, et al., *J. Control. Release* **2004**, 98, 195–207.

78 N. Shi, R.J. Boado, W.M. Pardridge, *Pharm. Res.* **2001**, 18, 1091–1095.

79 N. Shi, Y. Zhang, C. Zhu, et al., *Proc. Natl. Acad. Sci. USA* **2001**, 98, 12754–12759.

80 Y. Zhang, Y.F. Zhang, J. Bryant, et al., *Clin. Cancer Res.* **2004**, 10, 3667–3677.

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Enhancement of immune responses by DNA vaccination through targeted gene delivery using mannosylated cationic liposome formulations following intravenous administration in mice

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Abstract

The present study investigated the potency of the mannosylated cationic liposomes (Man liposomes) that we have developed in novel DNA vaccine carrier. Ovalbumin (OVA) was selected as a model antigen for vaccination; accordingly, OVA-encoding pDNA (pCMV-OVA) was constructed to evaluate DNA vaccination. The potency of the Man liposome/pCMV-OVA complex was compared with naked pCMV-OVA and that complexed with DC-Chol liposomes. In cultured mouse peritoneal macrophages, MHC class I-restricted antigen presentation of the Man liposome/pCMV-OVA complex was significantly higher than that of naked pCMV-OVA and that complexed with DC-Chol liposomes. After intravenous administration, OVA mRNA expression and MHC class I-restricted antigen presentation on $CD11c^+$ cells and inflammatory cytokines, such as TNF-α, IL-12, and IFN-γ, that can enhance the Th1 response of the Man liposome/pCMV-OVA complex were higher than that of naked pCMV-OVA and that complexed with DC-Chol liposomes. Also, the spleen cells from mice immunized by intravenous administration of the Man liposome/pCMV-OVA complex showed the highest proliferation response and IFN-γ secretion. These findings suggest that the targeted delivery of DNA vaccine by Man liposomes is a potent vaccination method for DNA vaccine therapy.

Keywords: DNA vaccine; Gene delivery; Mannosylated liposome; Cationic liposome; Plasmid DNA; Targeting

DNA vaccination, the administration of DNA-encoding antigen gene into the body, is of great interest in gene therapy as a means of immunotherapy against 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 [1–3]. This cell-mediated immune response plays a crucial role in the immune response against cancer and infectious diseases [4]. Recent immunological studies have demonstrated that gene transfection and subsequent activation of dendritic cells are key events in the development of immunity following DNA vaccination [5]. Antigen-presenting cells (APCs), especially dendritic cells (DCs), process peptide epitopes in the expressed antigen in the context of both class I and class II MHC molecules resulting in the induction of cytotoxic T lymphocytes (CTL) with the help of $CD4^+$ T cells [6,7]. Consequently, high transfection efficiency into DCs is essential for efficient DNA vaccination. In the early 1990s, Wolff et al. [8] demonstrated that sustained and efficient gene transfection could be achieved following the intramuscular administration of naked plasmid DNA (pDNA). Therefore, many attempts have been made to use naked pDNA-based immunization to produce humoral and cellular immunity and demonstrate its potency in non-human primates. However, recent clinical trials have shown that the immune response following topical injection of naked pDNA solution is insufficient [9,10], indicating that further improvements in the transfection efficiency involving some pharmaceutical modification are needed for DNA vaccine therapy.

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So far, many kinds of vectors, both viral and non-viral, have been developed to enhance transfection activity to various cells [11–15]. Although virus-based vectors are known to exhibit high transfection efficiency to DCs, their immunogenicity and toxicity restricts their application to ex vivo DC therapy. Non-viral vectors, such as cationic polymers and cationic liposomes, have seen a great advance clinically due to their low toxicity, low immunogenicity, and ease of preparation.

Perrie et al. [16] reported that cationic liposomes enhance gene expression in the draining lymph node, which contains many APC populations, after intramuscular administration as well as enhancing the antigen-specific antibody response. Furthermore, other investigators have also demonstrated that cationic liposomes enhance the CTL response to antigens, such as hepatitis C virus, human immunodeficiency virus, and influenza antigen [17–19]. Although the adjuvant effect of cationic liposomes has been widely accepted, the lack of cell-specificity of cationic liposomes after in vivo application is regarded as limiting their transfection efficiency to APCs and the resulting adjuvant efficiency. For efficient gene therapy, non-viral vectors offer the major advantages of sustained effect, high-level transgene expression with minimal toxicity, and immunological side effects. It is well known that APCs express high levels of mannose receptors having a high capacity for the uptake of antigens that have mannose residues. Therefore, one promising approach for efficient gene delivery to APCs is attachment of mannose residues to cationic liposomes for cell-selective gene transfection.

Recently, we synthesized a novel mannosylated cationic cholesterol derivative{(cholesten-5-yloxy-*N*-(4-((1-imino-2-D-thiomannosyl-ethyl)amino)butyl)formamide) (Man-C4-Chol)} for the preparation of mannosylated liposomes (Man liposomes) [20,21]. Man-C4-Chol exhibits bi-functional properties, i.e., an imino group for binding to pDNA via electrostatic interaction and a mannose residue for the cell surface receptors in APCs. Therefore, a high density of mannose residues can be provided on the liposome surface without affecting the binding ability of the cationic liposomes to DNA in these mannosylated cholesterol derivatives. In fact, we have reported that Man-C4-Chol mixed with dioleoylphosphatidylethanolamine (DOPE), which is a pH-sensitive lipid that accelerates the endosomal escape of pDNA [22], results in liposomes that can deliver firefly luciferase-encoding pDNA (pCMV-Luc), a conventional model gene for evaluating gene transfection. This delivery is selective for APCs via mannose receptor-mediated endocytosis after intravenous or intraportal injection into mice [20,23].

The Man liposome formulation allows development of a DNA vaccine with suitable pharmaceutical properties for APC targeting under in vivo conditions; therefore, this carrier system is expected to improve the immune response of this novel DNA vaccine. We selected ovalbumin (OVA) as a model antigen for DNA vaccination; accordingly, OVA encoding pDNA (pCMV-OVA) was constructed for the evaluation of DNA vaccination. The potency of the Man liposome/pCMV-OVA complex was compared with that of naked pCMV-OVA and that complexed with 3β [*N*-(*N'*-*N'*-dimethylaminoethane)-carbamoyl]cholesterol (DC-Chol) liposomes [24].

Materials and methods

Materials. Cholesteryl chloroformate, Hepes, and OVA were obtained from Sigma Chemicals (St. Louis, MO, USA). Diphosphatidylethanolamine (DOPE) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). *N*-(4-aminobutyl)carbamic acid *tert*-butyl ester was obtained from Tokyo Chemical Industry (Tokyo, Japan). Interleukin (IL)-2, IL-12, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α ELISA kits were purchased from Genzyme Techne (Minneapolis, MN, USA). pVAX I, fetal bovine serum (FBS), and Opti-MEM I were obtained from Invitrogen (Carlsbad, CA, USA). Anti-CD11c monoclonal antibody (N418)-labeled magnetic beads were purchased from Miltenyi Biotec (Auburn, CA, USA). Alamar-Blue was purchased from TRECK Diagnostic Systems (West Sussex, UK). RPMI 1640 and thioglycollate medium were purchased from Nissui Pharmaceutical (Tokyo, Japan). Nucleic Acid Purification Kit MagExtractor-RNA- was purchased from TOYOBO (Osaka, Japan). 1st strand cDNA synthesis kit for RT-PCR(AMV), Lightcycler fast-start DNA master hybridization probes, and Lightcycler-Primer/Probes set for mouse β-actin were purchased from Roche diagnostics (Indianapolis, IN, USA). Primers/Probes for OVA were purchased from NIHON Gene Research Lab's (Sendai, Japan). All other chemicals were of the highest purity available.

Mice and cell line. Female ICR mice (4–5 weeks) and female C57BL/6 mice (6–8 weeks old) were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). All animal experiments were carried out in accordance with the Principles of Laboratory Animal Care as adopted and promulgated by US National Institutes of Health and the Guideline for Animal Experiments of Kyoto University. CD8OVA1.3 (kindly provided by Dr. C.V. Harding, Case Western Reserve University, Cleveland, OH, USA) [25] is a T cell hybridoma cell, which is specific for OVA (257–264)-kb.

Synthesis of Man-C4-Chol. Man-C4-Chol was synthesized as described previously [21]. In brief, *N*-(4-aminobutyl)-(cholesten-5-yloxyl)formamide (C4-Chol) [26] was synthesized from cholesteryl chloroformate and *N*-(4-aminobutyl)carbamic acid *tert*-butyl ester. The C4-Chol was reacted with 5 equivalents of 2-imino-2-methoxyethyl-1-thiomannoside [27] in pyridine containing 1.1 equivalents of triethylamine for 24 h. After evaporation of the reaction mixture in vacuo, the resultant material was suspended in water and dialyzed against water for 48 h and then lyophilized.

Construction and preparation of pCMV-OVA. pCMV-OVA was constructed by subcloning the *Eco*RI chicken egg albumin (ovalbumin) cDNA fragment from pAc-neo-OVA, which was kindly provided by Dr. M.J. Bevan (University of Washington, Seattle, WA, USA) into the polylinker of pVAX I. pCMV-OVA was amplified in the *Escherichia coli* strain, DH5α, then isolated, and purified using a Qiagen Plasmid Giga Kit (Qiagen GmbH, Hilden, Germany). For immunization experiments, the endotoxin in pCMV-OVA solution was removed by the Triton X-114 method.

Preparation of DC-Chol and Man liposomes. Man-C4-Chol or DC-Chol was mixed with DOPE in chloroform at a molar ratio of 3:2 and the mixture was dried, vacuum desiccated, and resuspended in sterile 20 mM Hepes buffer (pH 7.8) or 5% dextrose solution in a sterile test