plasmid DNA/Man–liposome complex with macrophages. Although serum proteins are reported to nonspecifically block the transfection activity of the plasmid DNA complex [38], this was not the case for this experiment since the addition of BSA had no significant effect on the efficiency. The net negative charge of the complex might explain the differences from the previous reports. In the previous studies, we have shown that MBP bind to DSPC/Chol/Man-C4-Chol liposome via the mannose unit of Man-C4-Chol [22]. It is reasonable that MBP could bind to this cationic mannosylated liposome (DOPE/Man-C4-Chol). These results suggest that serum MBP specifically binds to the mannose moieties presented on the complex and suppresses its cellular uptake. Such an interaction could be an obstacle to mannose receptor-mediated in vivo gene transfer to mannose receptor-positive cells by using mannosylated gene carriers. The addition of 10 μg MBP to plasmid DNA complex greatly decreased gene expression to about 10% of the control value (Fig. 3), whereas the cellular association of the [^{32}P] plasmid DNA complex was about 70% of the control value when 10 μg MBP was added (Fig. 5). Although the electrical charge of the complex was controlled to be negative, plasmid DNA complex could nonspecifically bind with the cells and such a binding might not be inhibited by MBP. Therefore, the discrepancy between the degrees of inhibition by MBP in gene expression and in the association suggests that gene expression in macrophages occur mainly through the mannose receptor-mediated endocytosis. Further studies are needed to confirm this assumption.

These results seem to be inconsistent with previous results obtained with bare mannosylated liposomes composed of Man-C4-chol, distearoyl phosphatidylcholine (DSPC), and cholesterol with neutral charge and without plasmid DNA. In this case, serum MBP enhanced its cellular uptake by macrophages [22]. This could be due to differences in the lipid composition and the charge on the liposomes as well as complex formation with DNA. Although the structure, functions, and manner of binding to the simple sugar of MBP have been extensively studied [39,40], there is little information about the relationship between the characteristics of the mannosylated ligands and the accelerated (opsonins) or suppressed (disopsonins) efficiency of the MBP complex. The critical features of the ligands, such as their size, charge, shape, and the mechanism of the inhibition/stimulation of MBP on the uptake of mannosylated ligands, thus, remain to be elucidated.

In summary, the specific delivery of plasmid DNA to macrophages via mannose receptors was partially inhibited by serum MBP. This inhibition is provoked by the specific recognition of mannose moieties on the plasmid DNA/Man–liposome complex by serum MBP. Since the recognition characteristics of these two mannose-specific lectins, i.e., the mannose receptor and serum MBP, differ [23], tight control of the composition and structure of the plasmid DNA/Man–liposome complex would lead to an increase in the in vivo transfection efficiency in cells possessing mannose receptors.

Acknowledgements

We would like to thank Professor Toshisuke Kawasaki and Dr. Kazuhide Uemura for their helpful advice on the separation of serum mannan-binding protein. This work was supported in part by a Grant-In-Aid for Scientific Research from the Ministry of Education, Science, and Culture, Japan.

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Journal of Drug Targeting, 2002 Vol. 10 (2), pp. 99–104

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healthsciences

Influence of Physicochemical Properties on Pharmacokinetics of Non-viral Vectors for Gene Delivery

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(Received 25 September 2001)

The influence of physicochemical properties on the *in vivo* pharmacokinetics of gene delivery vectors after systemic administration is reviewed based on our studies. We have been studying the development of DNA delivery systems, such as plasmid DNA complexed with cationic polymers (polyplexes) and cationic liposomes (lipoplexes). Even if target-recognizable ligand is incorporated into the system, the overall physicochemical properties, notably size and charge, are predominant factors influencing *in vivo* disposition characteristics of the vector. Based on this consideration, liver cell-specific carrier systems via receptor-mediated endocytosis were successfully developed by optimizing physicochemical characteristics. In conclusion, rational design of gene delivery vectors requires an understanding of their pharmacokinetics in relation to the physicochemical properties. Optimization of the physicochemical properties is important for successful *in vivo* gene delivery by non-viral vectors.

Keywords: *In vivo* gene delivery; Pharmacokinetics; Physicochemical properties; Polyplex; Lipoplex; Receptor-mediated endocytosis

INTRODUCTION

In vivo gene therapy requires the development of carriers (vectors) which can deliver therapeutic genes to a specific cell population via systemic or local administration and efficiently express encoded proteins at the target site. The vectors for gene delivery should be safe for repeated use and provide reproducible levels of the gene products. Although viral systems such as adenoviral vectors are potentially very efficient, non-viral gene delivery systems have some advantages in that they are less toxic, less immunogenic, and easier to prepare.

Among the modalities of gene delivery, intravascular gene delivery via systemic (intravenous) or local (intraarterial) routes have a special advantage from the therapeutic point of view if cell-specific or targeted gene delivery and expression can be achieved. Receptor-mediated gene delivery is one of the most useful strategies for this purpose.

Non-viral vectors include purified plasmid DNA itself (naked DNA) or the polyion complexes formed between polycationic carriers such as cationic liposomes (i.e. lipoplexes) and polymers (polyplexes). Cell-specific gene delivery can be promoted by incorporating target recognizable ligand into the vectors. A typical example of such a vector would be asialoorosomucoid-poly(L-lysine) (AsOR-PLL) conjugate/DNA complexes, developed in pioneer work by Wu and coworkers (Wu and Wu, 1988; Wilson *et al.*, 1992), for hepatocyte-specific plasmid DNA delivery after intravenous injection. Directly glycosylated PLL derivatives have also been used for successful *in vivo* gene delivery in a cell-specific manner (Perales *et al.*, 1994; Ferkol *et al.*, 1996).

It is very important to develop sophisticated cell-specific vector systems, which can be recognized by the receptors on the target cells. However, in addition to this biological functionality, the physicochemical properties of the vector are also critically important. The availability of the gene at the target site is primarily decided by the behavior in the whole body or pharmacokinetics of the vector, which are mainly dictated by its physicochemical properties such as size and charge (zeta-potential). In spite of extensive development of targeting vectors, little attention has been paid to this aspect, meaning that

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ISSN 1061-186X print/ISSN 1029-2330 online © 2002 Taylor & Francis Ltd
DOI: 10.1080/10611860290016694

systems have not been optimized. Based on these considerations, we have developed a variety of polyplexes (Nishikawa *et al.*, 1998; 2000a,b) and lipoplexes (Kawakami *et al.*, 2000a,b) aiming at cell-specific plasmid DNA delivery after systemic administration. In this article, we will briefly review the influence of physicochemical properties of gene delivery vectors such as naked DNA, polyplexes and lipoplexes on their pharmacokinetics based on our recent studies.

GENERAL CONSIDERATIONS

For a rational design of *in vivo* gene delivery vectors, various aspects should be considered from the viewpoint of pharmacokinetics (Takakura and Hashida 1996; Takakura *et al.*, 1996). The fate of injected gene delivery vectors is dictated mainly by their overall physicochemical properties, even if target-specific ligands are incorporated. After systemic administration of gene delivery vectors, a series of pharmacokinetic events occur in the intravascular space before reaching the target site. Only the gene delivery vectors that can survive in these processes have a chance to distribute to and enter the target cells in functional form. However, due to low capillary permeability of most organs, the distribution of blood-born gene delivery vectors is basically restricted to the intravascular space and no significant extravasation can be expected. Possible targets would therefore be some tissues with higher capillary permeability such as the liver and tumors. However, prolonged circulation in the blood stream would be required for satisfactory extravasation of the vectors within these tissues.

Figure 1 schematically summarizes the major barriers in the body for hepatocyte-specific gene delivery via the asialoglycoprotein (ASGP) receptor, as an example, after systemic administration of lipoplexes or polyplexes. Immediately after injection, cationic vectors may form aggregates due to interaction with blood components including serum proteins and blood cells, and may adsorb to the vascular walls via electrostatic force. Aggregated vectors may then lead to embolization of capillaries. In the case of intravenous injection, large aggregates would be trapped in the capillary bed in the lung, the first-pass organ from this administration route. Non-specific uptake by non-target cells including phagocytes like Kupffer cells and other macrophages may also take place. For receptor-mediated uptake, the vector must pass through the capillary wall in the liver and therefore its size must be smaller than that of capillary fenestra (about 200 nm) for efficient extravasation. Thus the physicochemical properties of the vector significantly affect all the distribution processes in the body following systemic injection before reaching the target cell surface. It is therefore essential to control their physicochemical properties in the development of cell-specific gene delivery systems.

PHARMACOKINETICS OF NAKED PLASMID DNA AFTER SYSTEMIC INJECTION

It would be useful to understand the pharmacokinetic properties of naked plasmid DNA in relation to its physicochemical properties, to enable rational design of polyplexes and lipoplexes. Plasmid DNA is double-stranded DNA, a huge polyanion with a molecular weight of about

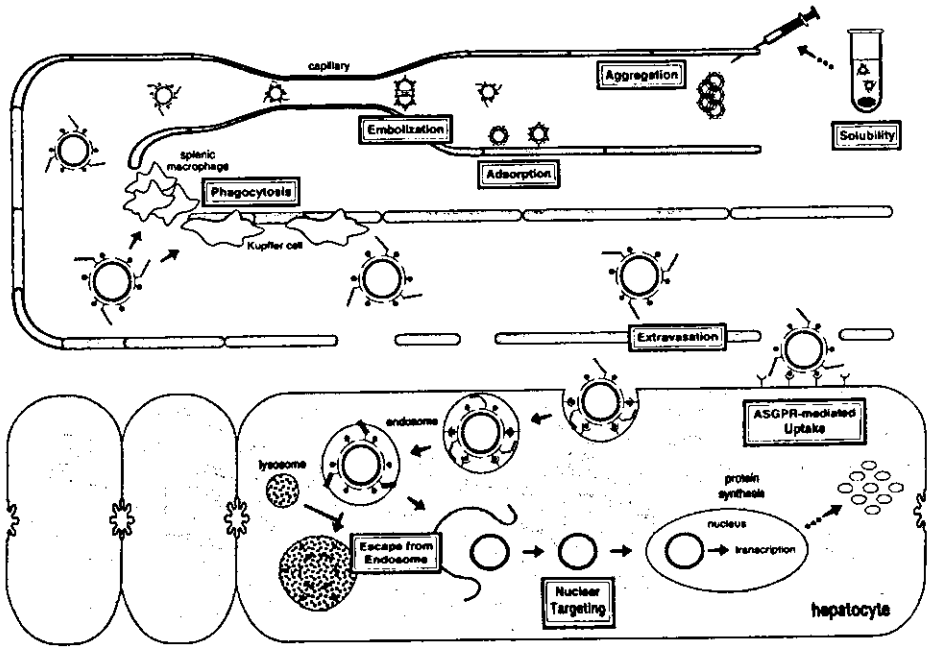

FIGURE 1 Schematic representation of the processes in the body and a target cell (hepatocyte) after intravenous injection of a non-viral gene delivery vector. ASPGR; asialoglycoprotein receptor.

3–4 million, whose pharmacokinetic properties after intravenous injection can be analyzed on the basis of physiological pharmacokinetic model (Takakura *et al.*, 1996; Nishikawa *et al.*, 1996). We studied the disposition of a plasmid DNA radiolabeled with ^{32}P following intravenous injection into mice (Kawabata *et al.*, 1995; Kobayashi *et al.*, 2001).

After intravenous injection ^{32}P-labeled plasmid DNA was rapidly eliminated from the circulation and predominantly taken up by the liver nonparenchymal cells, such as Kupffer and endothelial cells, with no significant gene expression observed in this organ. Rapid degradation by nucleases in serum and tissues were also observed. Pharmacokinetic analysis has demonstrated that the hepatic uptake clearance of DNA at lower dose is similar to the plasma flow rate in the liver, suggesting highly effective first-pass elimination by this organ. Involvement of a specific mechanism was demonstrated since the hepatic uptake of [^{32}P] DNA was dramatically inhibited by cold plasmid DNA, calf thymus DNA and some polyanions (polyinosinic acid, dextran sulfate, heparin etc.), but not by others (polycytidylic acid, chondroitin sulfate etc.). Further studies including *in vitro* experiments using cultured peritoneal macrophages from class A macrophage scavenger (SRA) knockout mice have demonstrated that plasmid DNA uptake is mediated by a receptor similar to, but distinct from, SRA (Yoshida *et al.*, 1996; Takagi *et al.*, 1998; Takakura *et al.*, 1999), a conclusion supported by another recent study using SRA knockout mice (Zhu *et al.*, 2001).

Recently, it has been found that a high level of gene expression can be easily obtained in mice by simple injection of naked pDNA into the tail vein with a large volume of saline at a high velocity (Zhang *et al.*, 1999; Liu *et al.*, 1999). This is the so-called hydrodynamics-based transfection procedure. The hydrodynamics-based procedure is very attractive since selective and significant gene expression can be obtained in the liver without any DNA carriers. This procedure has been applied as a simple and efficient *in vivo* transfection method for screening genes in mice (Zhang *et al.*, 2000; He *et al.*, 2000). Recently we carried out a pharmacokinetic study after tail vein injection of [^{32}P] plasmid DNA with this procedure (Kobayashi *et al.*, 2001). We found that the hepatic uptake profile was similar after normal injection to that after intravenous injection of naked pDNA with a large volume of saline at a high velocity. Nevertheless, the hepatic uptake in the hydrodynamic approach was not inhibited by prior administration of polyanions, including poly I, dextran sulfate and heparin. The hydrodynamics-based procedure also resulted in marked gene expression in the liver, which again was not inhibited by prior administration of polyanions. These results indicate that plasmid DNA uptake and subsequent gene expression in the liver would be a nonspecific process. It has been demonstrated that hepatocytes are responsible for the significant gene expression in this organ (Li *et al.*, 1999). Thus this particular procedure can modulate the mode of hepatic delivery of naked plasmid DNA after intravenous injection. It is feasible that the procedure causes backflow of injectate into the hepatic vein and thence into the liver parenchyma, displacing the blood and avoiding exposing the DNA to nucleases, serum proteins or other possibly inhibitory components of the serum.

INFLUENCE OF PHYSICOCHEMICAL PROPERTIES ON PHARMACOKINETICS OF POLYPLEXES

Cell-specific gene delivery can be achieved after systemic administration by using polyplexes having ligands that are selectively recognized by the target cells. Polycations such as poly(L-lysine) (PLL) are linked to a tissue-specific ligand, and then bind to DNA via electrostatic interactions. The resulting complexes retain their ability to interact specifically with cognate receptors on the target cells leading to the receptor-mediated internalization of the complex into the cell.

We attempted to design polyplexes based on the considerations of the pharmacokinetics of the vector in relation to their physicochemical properties (Nishikawa *et al.*, 1998). Galactosylated (Gal-PLL) derivatives were synthesized as carrier candidates using 2-imino-2-methoxyethyl 1-thioglycoside by the method of Lee *et al.* (1976). Gal-PLL with different sugar modification ratios were prepared using PLL with average molecular weights of 1800, 13,000 and 29,000. Complex formation between the PLL derivatives and plasmid DNA were optimized by characterization of the physicochemical properties of the complex, i.e. particle size and zeta-potential. The size of the polyplexes should be less than 200 nm for efficient extravasation across the fenestration of the sinusoid (about 200 nm). As far as the zeta-potential is concerned, we decided to avoid strong cationic charges because it may lead to undesirable binding to blood components and nonspecific biodistribution via electrostatic interaction. We selected several types of polyplexes for *in vivo* studies which have diameters of 200 nm or less weak negative charges on their surface.

After intravenous injection of [^{32}P] plasmid DNA/Gal_{13}-$PLL_{13,000}$ (PLL with molecular weight of 13,000 and 13 galactose residues) and [^{32}P] plasmid DNA/Gal_{26}-$PLL_{29,000}$ into mice, almost 80% of the radioactivity rapidly accumulated in the liver. Hepatocyte-specific localization in the liver of the injected plasmid was also confirmed by collagenase perfusion experiments. The ASGP receptor-mediated endocytosis of the Gal-PLL complex was also confirmed by competitive inhibition experiments using galactosylated albumin. On the other hand, [^{32}P] plasmid DNA/Gal_5-PLL_{1800} and [^{32}P] plasmid DNA/Gal_5-$PLL_{13,000}$ resulted in insufficient delivery to the liver, suggesting that both the molecular weight of PLL and the degree of galactose modification determine the hepatic targeting of plasmid DNA. Significant gene expression was observed only after intravenous injection

of Gal-PLL complexes exhibiting efficient delivery potential. Thus, a rational design of polyplexes is possible when the influence of physicochemical properties on their pharmacokinetics is taken into considerations. A similar strategy was also applied to the design of mannosylated PLL (Man-PLL) as a liver non-parenchymal specific carrier (Nishikawa *et al.*, 2000b).

Although controlled biodistribution of polyplexes through optimization of their physicochemical properties was shown, the gene expression level in the liver was not very intense. Therefore, in order to improve intracellular pharmacokinetics, we designed a multifunctional carrier that controls both the tissue and intracellular distribution of DNA (Nishikawa *et al.*, 2000a). A cationic polymer, poly(L-ornithine) (pOrn), was modified first with galactose, then with a membrane fusogenic peptide (mHA2) to obtain Gal-pOrn-mHA2. The fusogenic peptide can facilitate DNA release from endosomes/lysosomes into cytoplasm after internalization. The carrier was mixed with DNA to form polyplex having a relatively small size (about 130 nm), and then the polyplex was intravenously injected into mice via the tail vein. Then the biodistribution and gene expression after intravenous injection of the polyplex (10 μg DNA/mouse) were examined. After injection of [^{32}P]DNA/Gal-pOrn-mHA2, about 60% of the radioactivity was recovered in the liver, mostly in parenchymal cells. A large amount (81 ng/g tissue) of transgene product (luciferase) was detected in the liver of mice injected with DNA/Gal-pOrn-mHA2, which was 280-fold greater than that obtained with DNA/DOTMA: Chol liposomes (50 μg DNA). Prior administration of galactosylated albumin reduced the gene expression to 1/100, indicating the ASGP receptor-mediated gene transfer in liver parenchymal cells, i.e. hepatocytes. The luciferase activity in hepatocytes contributed more than 95% of the total activity in all the tissues examined. Thus, highly-specific and relatively efficient gene transfer into hepatocytes can be achieved by the intravenous injection of polyplexes employing the multi-functional gene carrier.

PEGylation appears to be a useful method to prolong blood circulation of polyplexes after intravenous injection (Ogris *et al.*, 1999; Kircheis *et al.*, 2001). This technique has been applied to *in vivo* gene delivery to tumors following systemic injection. Incorporation of the receptor binding ligand transferrin into DNA/polyethylenimine (PEI) complexes was found to enhance gene transfer into tumor cell lines in a receptor-dependent manner. Ogris *et al.* (1999) investigated the *in vitro* and *in vivo* properties of DNA/PEI (800 kDa) complexes before and after covalent coupling of poly(ethylene glycol) (PEG). The positively charged non-PEGylated polyplexes form aggregates during incubation with plasma or erythrocytes due to electrostatic interaction with the blood components. PEGylation of the polyplexes strongly reduces plasma protein binding and erythrocyte aggregation. Furthermore, PEGylated polyplex size was stabilized and had a reduced surface charge. Prolonged circulation in the blood of the PEGylated polyplexes was also observed when injected intravenously. In contrast to non-PEGylated polyplexes which directed gene transfer primarily to the lung, PEGylated polyplexes mediated reporter gene transfer to the tumor without significant toxicity after intravenous injection into tumor bearing mice.

INFLUENCE OF PHYSICOCHEMICAL PROPERTIES ON PHARMACOKINETICS AND LIPOPLEXES

Recent studies have demonstrated that the intravenous injection of lipoplexes can exhibit a significant *in vivo* transfection activity after intravenous injection, particularly in the lung, when cholesterol (Chol) is used as a helper lipid (Templeton *et al.*, 1997; Liu *et al.*, 1997; Li *et al.*, 1998; 1999; Meyer *et al.*, 2000). Although passive targeting of lipoplexes to the lung can be easily achieved since the capillary endothelial cells in this organ offer the first contact with lipoplexes injected intravenously, targeted gene delivery to other organs would be difficult, especially by receptor-mediated mechanisms. Immediately after intravenous injection, the physicochemical properties of lipoplexes, should be modified by the interaction with blood components such as serum proteins (Li *et al.*, 1999) and erythrocytes (Sakurai *et al.*, 2001), which may lead to impaired transfection ability.

Besides polyplexes as described above, we have also developed a novel lipoplex system aiming at hepatocyte-specific gene delivery via ASGP receptor-mediated endocytosis (Kawakami *et al.*, 1998). We synthesized novel galactosylated cholesterol derivatives, cholesten-5-yloxy-*N*-(4-((1-imino-2-β-D-thiogalactosylethyl)amino) alkyl) formamide, for hepatocyte-directed gene carriers, demonstrating that the liposomes containing this galactolipid showed higher transfection activities than 3β[*N'*,*N'*,*N'*-dimethylaminoethane)carbamoyl]cholesterol (DC-Chol) liposomes in human hepatoma cells (HepG2). Based on the *in vitro* finding, we studied the *in vivo* pharmacokinetics and gene transfer for lipoplexes containing one of the derivatives, cholesten-5-yloxy-*N*-(4-((1-imino-2-β-D-thiogalactosylethyl)amino)butyl) formamide (Gal-C4-Chol), in relation to lipid composition and charge ratio (Kawakami *et al.*, 2000b). Galactosylated cationic liposomes containing *N*-[1-(2,3-dioleyloxy)propyl]-*n*,*n*,*n*-trimethylammonium chloride (DOTMA), Gal-C4-Chol and cholesterol (Chol) and other liposomes with similar particle size (130–150 nm) were prepared. Following intravenous injection of lipoplexes, the highest gene expression was observed in the lung while the gene expression in the liver was much lower in all the formulations tested.

On the other hand, after intraportal injection, a markedly higher gene expression in the liver was observed following injection of lipoplexes containing DOTMA/Chol/Gal-C4-Chol(1:0.5:0.5) and DOTMA/Gal-C4-Chol(1:1) liposomes. The effect was one order of magnitude higher than naked DNA and DOTMA/

Chol(1:1) liposomes. Pre-exposing with galactosylated bovine serum albumin significantly reduced the hepatic gene expression. As far as the charge ratio of DOTMA/Chol/Gal-C4-Chol(1:0.5:0.5) liposomes to plasmid DNA(1.6–7.0) was concerned, complexes with charge ratios of 2.3–3.1 produced maximal gene expression in the liver, whereas higher ratios resulted in enhanced expression in the lung. These results indicate that, by optimizing lipid composition and charge ratio, galactosylated lipoplexes allow superior *in vivo* gene transfection in the liver via ASGP receptor-mediated endocytosis.

Furthermore, we applied the same strategy to *in vivo* gene delivery through mannose receptors on liver non-parenchymal cells including Kupffer and sinusodial endothelial cells (Kawakami *et al.*, 2000a). A novel mannosylated cholesterol derivative, cholesten-5-yloxy-*N*-(4-((1-imino-2-β-D-thiomannosyl-ethyl)amino)butyl)formamide (Man-C4-Chol), was synthesized. In this case, intravenous injection of lipoplexes containing the derivative was somewhat successful and a high gene expression was observed in the liver even after intravenous injection of Man-C4-Chol/DOPE(6:4) liposomes, whereas DC-Chol/DOPE(6:4) liposomes only showed marked expression in the lung. The gene expression with Man-C4-Chol/DOPE(6:4) liposome/DNA complexes in the liver was observed preferentially in the non-parenchymal cells and was significantly reduced by predosing with mannosylated bovine serum albumin. The gene expression in the liver was greater following intraportal injection. These results suggest that plasmid DNA complexed with mannosylated liposomes exhibits high transfection activity due to recognition by mannose receptors *in vivo*. We observed different transfection activities between the two types of lipoplexes containing galactose and mannose moieties after intravenous injection. The difference in the anatomical locations of target cells, i.e. the liver parenchymal and non-parenchymal cells, may be, in part, responsible for the phenomenon. Extravasation of the lipoplexes is not required for the latter target.

PEGylation also has been shown to be a useful method to improve pharmacokinetics of lipoplexes after systemic application (Monck *et al.*, 2000; Tam *et al.*, 2000). Plasmid DNA can be encapsulated in lipid particles (SPLP, "stabilized plasmid lipid particles") of approximately 70 nm diameter composed 1,2-dioleoyl-3-phosphatidylethanolamine (DOPE), the cationic lipid *N,N*-dioleoyl-*N,N*-dimethylammonium chloride (DODAC) and poly(ethylene glycol) conjugated to ceramide (PEG-Cer). It is shown that SPLP exhibit long circulation lifetimes (circulation half-life >6 h) following intravenous injection in a murine tumor model resulting in accumulation of up to 3% of the total injected dose and concomitant reporter gene expression at a distal (hind flank) tumor site. In contrast, intravenous injection of naked plasmid DNA or plasmid DNA-cationic liposome complexes did not result in significant plasmid delivery to the tumor site or gene expression at that site. Furthermore, SPLP showed no marked toxicity. It is concluded that SPLP exhibit properties consistent with potential utility as a nontoxic systemic gene therapy vector.

CONCLUSION

In conclusion, rational design of non-viral gene delivery vectors, such as polyplexes and lipoplexes, requires an understanding of their pharmacokinetics in relation to the physicochemical properties, notably size and charge. Optimization of the physicochemical properties is important for successful *in vivo* gene delivery by non-viral vectors based upon active targeting strategies as well as passive ones.

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19 Basic pharmacokinetics of oligonucleotides and genes

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INTRODUCTION

The ultimate goal of gene therapy is to cure inheritable and acquired diseases in a straightforward manner by correcting abnormalities in genes. Various protocols have been employed to express gene product including the addition of the wild-type gene, correcting mutation in the gene, or suppressing undesirable gene products by blocking mRNA with complementary antisense oligonucleotide. Like conventional drugs and biologically active protein drugs, the administration of plasmid DNA (pDNA) and oligonucleotide (both are called 'gene drugs') directly to patients represents an ideal methodology for the treatment of a variety of diseases. Following *in vivo* administration, however, they encounter many hurdles that must be overcome for a successful therapy.

Biodistribution of a drug, which is administered externally as a free form or with a delivery system, is determined by its interaction with the body, based on the physicochemical and biological properties of the drug and the anatomical and physiological properties of the body. Therefore, drug targeting can be achieved by altering the properties of a drug with the drug delivery system (Senior, 1987; Takakura and Hashida, 1996; Nishikawa *et al.*, 1996), or by altering the properties of tissues, such as the osmotic opening of the blood-brain barrier (Robinson and Rapoport, 1987). Changing the route of administration, for example, using intraarterial injection to the target tissue instead of intravenous injection, is also a promising approach for improving the targeting efficiency of a drug (Hunt *et al.*, 1986). Similar strategies can be applied to a gene drug to deliver it to specific target tissues/cells. However, contrary to most conventional or protein drugs, pDNA and oligonucleotide should find a way to the intracellular space within the nucleus or cytoplasm where they have a chance to work.

Since Wolff *et al.* (1990) reported gene expression in skeletal muscle by a simple intramuscular injection of naked pDNA, it has widely been accepted that nucleic acids injected directly into muscle enter the cells. However, the way in which nucleic acids finally find a way to the nucleus after their cellular entry needs to be elucidated. Even for systemic administration, naked pDNA can produce a high level of transgene product when it is rapidly injected into the systemic circulation with a large-volume of solution (Zhang *et al.*, 1999; Liu *et al.*, 1999). However, a conventional intravenous injection of pDNA results in undetectable gene expression in major tissues (Kawabata *et al.*, 1995). Tissue and intracellular distribution of a gene drug would explain such differences as the level of

transgene product in the final output. To improve the distribution of gene drug after *in vivo* administration, various synthetic gene carriers, such as cationic lipids, polymers and peptides, have been developed. Gene drug, a negatively charged molecule, forms an electrostatic complex with cationic carriers and, within the body, such a complex interacts with various components and shows a complicated biodistribution profile.

Clear understanding of the biodistribution of pDNA and oligonucleotides, as well as their complex with delivery systems is a prerequisite to making a strategy for developing an *in vivo* gene transfer or modulation method. Pharmacokinetics translates the biodistribution properties of a gene drug into quantitative parameters, which can be compared with parameters obtained in a different condition, or with physiological parameters, such as blood flow and the rate of fluid-phase endocytosis. This chapter focuses on the pharmacokinetic evaluation of pDNA and oligonucleotides, in both free and complexed forms, administered systemically or locally.

WHOLE BODY PHARMACOKINETICS AFTER SYSTEMIC ADMINISTRATION

After systemic administration, a drug is generally delivered to tissues in the body through blood circulation. Therefore, the concentration of drug in plasma defines the rate of tissue uptake.

In vivo fate of drugs with large molecular weight such as a gene drug can be pharmacokinetically analyzed on the basis of a physiological pharmacokinetic model, as shown in Figure 19.1. Tissue uptake of a drug consists of an uptake

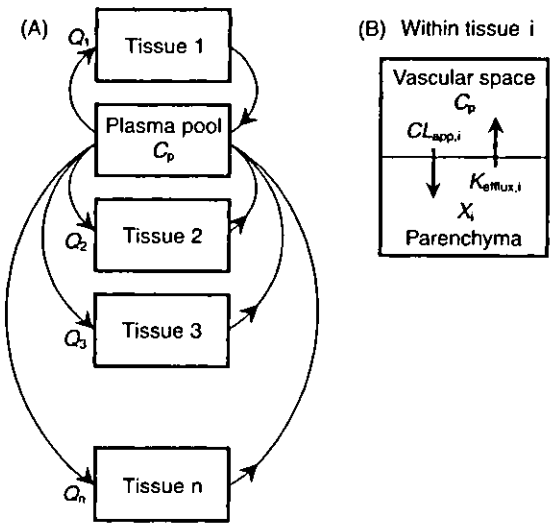

Figure 19.1 Physiological pharmacokinetic model for evaluating *in vivo* disposition of a macromolecular drug. (A) A multi-compartment model in which every tissue compartment is connected with the plasma pool by blood flow. (B) Tissue uptake of a drug from vascular space to tissue parenchyma.

from the plasma and an efflux from the tissue. When the tissue uptake rate is assumed to be independent on its concentration in the plasma and the efflux process follows the first-order rate kinetics, the change in its amount in a tissue with time can be described as follows:

$$\frac{dX_i}{dt} = CL_{app,i}C_p - k_{efflux,i}X_i \tag{1}$$

where X_i(μg) represents an amount of the drug in tissue i after administration, C_p (μg/mL) is its concentration in plasma, $CL_{app,i}$ (mL/h) expresses an apparent tissue uptake clearance from plasma to tissue i, and $k_{efflux,i}$ (h^{-1}) represents an efflux rate from tissue i. In some cases, the efflux process from tissues can be negligible, which makes it easier to analyze the distribution properties of a drug. When biodistribution of a drug is traced by the counting of radioactivity, the use of residualizing radiolabel will fit to this simplification (Ali *et al.*, 1988; Deshpande *et al.*, 1990). Polymers that are hardly metabolized after cellular uptake also remain within tissues by which they are taken up, and this feature allows for the omission of efflux processes from tissues in the pharmacokinetic analysis. However, it is not the case for gene drugs such as pDNA and oligonucleotide; they are easily degraded after cellular uptake and, in most cases, radioactivity derived from radiolabeled gene drugs is released from the cells. Even though the efflux of the tracing material occurs, the efflux from tissues should be ignored within short early periods following administration. When the efflux from tissues can be ignored, Eq (1) is simplified to:

$$\frac{dX_i}{dt} = CL_{app,i}C_p \tag{2}$$

Integration of Eq (2) from time *0* to t_1 gives:

$$CL_{app,i} = \frac{X_{i,t_1}}{\int_0^{t_1} C_p dt} = \frac{X_{i,t_1}}{AUC_{p,0-t_1}} \tag{3}$$

where $AUC_{p,0-t_1}$ (μg h/mL) is an area under the plasma concentration-time curve of a drug from time 0 to t_1. Its elimination profile from the plasma could be expressed as a function of one or more exponentials in many cases when AUC_p values at any time point can be calculated by fitting an equation to experimental data using a pharmacokinetic analysis program such as MULTI (Yamaoka *et al.*, 1981). According to Eq (3), $CL_{app,i}$ is obtained as the slope when the amount in a tissue (X_i) is plotted against AUC_p.

Tissue uptake clearance is a useful parameter to characterize the *in vivo* distribution properties of a drug since it is independent of the drug concentration in plasma. However, when the tissue uptake process depends on the drug concentration in plasma and follows non-linear kinetics, the calculated $CL_{app,i}$ represents an average value of its time-dependent clearance for the overall experimental period (Nishikawa *et al.*, 1992).

$CL_{app,i}$ is a hybrid parameter of the plasma flow rate (Q, mL/h) to a tissue and intrinsic uptake clearance ($CL_{int,i}$, mL/h) of the tissue and also expressed as:

$$CL_{app,i} = \frac{Q\ CL_{int,i}}{Q + CL_{int,i}} \tag{4}$$

When $CL_{int,i}$ is much larger than Q, $CL_{app,i}$ comes close to Q and this value (plasma flow rate to a tissue) is the upper limit of $CL_{app,i}$ whatever specific and rapid uptake mechanism is involved in the tissue uptake.

Total body clearance (CL_{total}, mL/h) of a drug can be calculated using AUC_p for infinite time ($AUC_{p,\infty}$) and an administered dose (D) as follows:

$$CL_{total} = \frac{D}{AUC_{p,\infty}} \tag{5}$$

Since CL_{total} is the sum of tissue uptake clearances and urinary excretion clearance, CL_{total} is also expressed as:

$$CL_{total} = CL_{app,liver} + CL_{app,kidney} + \cdots + CL_{urine} \tag{6}$$

$$= CL_{target} + CL_{non\text{-}target} \tag{7}$$

where CL_{target} denotes the uptake clearance of target tissue and $CL_{non\text{-}target}$ represents the sum of clearances except for CL_{target}. In the case of a gene drug, its degradation within systemic circulation is also a factor determining its efficacy. After administration, the fraction of a drug delivered to a target (F_{target}) can be calculated using CL_{target}, $CL_{non\text{-}target}$ and the degradation clearance of a drug within systemic circulation (CL_{deg}, mL/h) as follows:

$$F_{target} = \frac{CL_{target}}{CL_{total} + CL_{deg}} = \frac{CL_{target}}{CL_{target} + CL_{non\text{-}target} + CL_{deg}} \tag{8}$$

Therefore, the potential of targeted delivery of a gene drug can be quantitatively explained by the parameters of CL_{target}, $CL_{non\text{-}target}$ and CL_{deg}. Eq (8) clearly indicates that an approach increasing CL_{target} and/or reducing $CL_{non\text{-}target}$ or CL_{deg} of a gene drug is suitable for achieving an efficient gene transfer (or gene suppression) at target site. Figure 19.2A shows F_{target} as a function of CL_{target} when the sum of $CL_{non\text{-}target}$ and CL_{deg} is held constant at either 0.1, 1 or 10 mL/h. An increase in CL_{target} from 0.01 to 0.1 mL/h significantly enlarges F_{target} only when the sum of $CL_{non\text{-}target}$ and CL_{deg} is smaller than 1 mL/h. Figure 19.2B indicates that the reduction of $CL_{non\text{-}target}$ or CL_{deg} increases F_{target} of a gene drug when CL_{target} equals to 0.1 mL/h. These simulation studies clearly show a reasonable strategy for targeted delivery of a gene drug. If an inefficient delivery of a gene drug to a target is due to its rapid uptake by non-target tissues or extensive degradation in the systemic circulation, the drug should be protected from those unfavorable processes with a vector system. When a gene drug circulates in plasma for a long time (i.e., having small CL_{total}), the use of a ligand specifically recognized by the target, for example, galactose for hepatocytes, will increase the amount of drug delivered to the target.

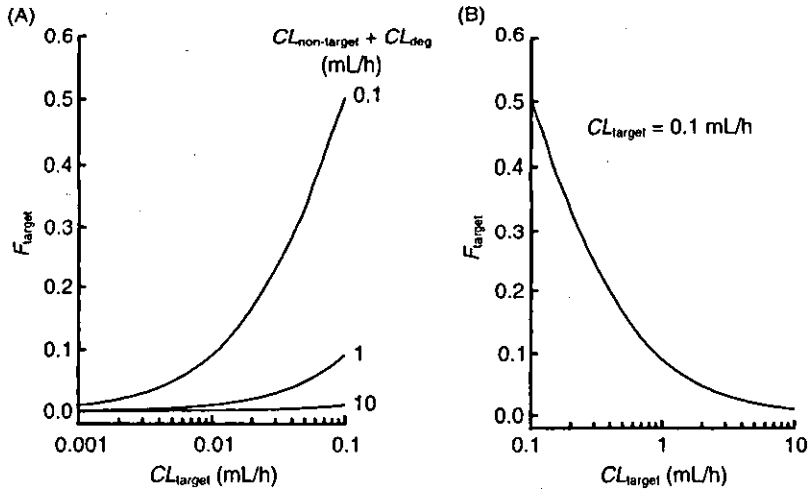

Figure 19.2 Effect of target (CL_{target}), non-target ($CL_{non\text{-}target}$) and degradation (CL_{deg}) clearances on a fraction of drug delivered to the target tissue (F_{target}) after systemic administration. The curves represent F_{target} simulated using Eq (8): (A) $CL_{non\text{-}target} + CL_{deg}$ is set at 0.1, 1 or 10 mL/h; (B) CL_{target} is fixed at 0.1 mL/h.

Figure 19.3 summarizes two major clearances governing the biodistribution of most macromolecular drugs – the hepatic uptake clearance and the urinary excretion clearance-of model compounds with diverse physicochemical characteristics following intravenous injection in mice. These plots clearly indicate that the biodistribution of macromolecules including pDNA and oligonucleotide is largely governed by their physicochemical properties such as molecular size and electrical charge.

Naked gene drug

pDNA

Systemic administration that does not require local, regional administration or surgical procedures is a desirable method for *in vivo* gene transfer. However, this route is one of the toughest ways for a gene drug to reach the target and express its function at the site because of a rapid removal by the liver and degradation.

The pharmacokinetics of naked pDNA in mice was examined using ^{32}P-labeled pDNA (Kawabata *et al.*, 1995). When naked ^{32}P-pDNA in 100 μL of saline solution was injected into mice at a dose of 1 mg/kg (about 25 μg/mouse), radioactivity was rapidly eliminated from plasma due to an extensive uptake by the liver, while it was not susceptible to glomerular filtration. In a different set of experiments, a rapid degradation by nucleases in serum was also observed. At this condition naked pDNA resulted in no gene expression in major organs (Mahato *et al.*, 1995a), possibly due to low DNA dose and its rapid degradation by nucleases. A pharmacokinetic analysis of the biodistribution of naked ^{32}P-pDNA showed that its hepatic uptake clearance (80 mL/h) is very close to the hepatic plasma flow rate of mice

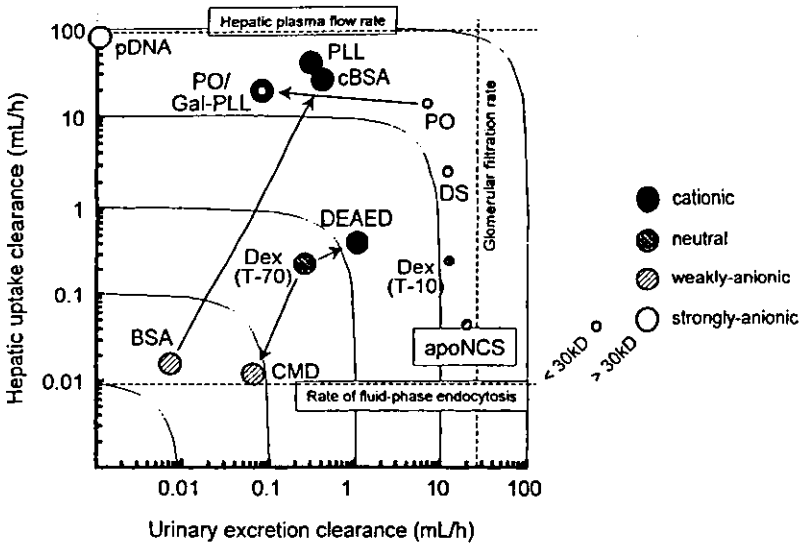

Figure 19.3 Apparent hepatic uptake clearance and urinary excretion clearance of model compounds in mice after intravenous injection. Strongly-anionic; pDNA (MW 2,850 kD), PO (20-mer phosphodiester oligonucleotide), DS (dextran sulfate, MW 8 kD). Weakly-anionic; apoNCS (apoprotein of neocarzinostatin, MW 12 kD), CMD (carboxymethyl-dextran derived from dextran T-70, average MW 70 kD), BSA (bovine serum albumin, MW 67 kD). Neutral; Dex(T-10) and Dex(T-70) (dextran T-10 and dextran T-70, average MW 10 and 70 kD, respectively). Cationic; DEAED (diethylaminoethyl-dextran derived from dextran T-70, average MW 70 kD), cBSA (cationized BSA, MW 70 kD), PLL (poly-L-lysine, average MW 40 kD). Ligand-complexed; PO/Gal-PLL (PO complexed with galactosylated PLL). Original data were published in following papers: pDNA, Kawabata *et al.* (1995); PO and PO/Gal-PLL, Mahato *et al.* (1997); DS, Takakura *et al.* (1994); apoNCS, CMD, BSA, Dex(T-10), Dex(T-70), DEAED, Takakura *et al.* (1990); cBSA, Fujita *et al.* (1995).

(85 mL/h for 25 g mouse) (Figure 19.3). Degradation clearance was also estimated from the stability data of pDNA in blood after intravenous injection to be 8.3 mL/h, about one-tenth of its hepatic uptake clearance. Thus, the pharmacokinetic analysis of the biodistribution of naked pDNA clearly shows that the rapid hepatic uptake, not its degradation in serum, is a major barrier for delivering pDNA to tissues, except for the liver.

On a point of gene expression following systemic administration of naked pDNA, Liu *et al.* (1999) and Zhang *et al.* (1999) reported that a rapid injection of a large volume of naked pDNA solution (for example, 5 μg pDNA in 1.6 mL saline solution for a 20 g mouse, which is almost equivalent to the total blood volume of the animal) into mice via the tail vein could induce an efficient gene transfer in internal organs, including the lung, spleen, heart, kidney and liver, with the highest level observed in the liver. Mechanisms for gene transfer by this approach have not been fully understood. However, it is proposed that the injected pDNA solution accumulates in the inferior vena cava, flows back to tissues directly linked to

this vascular system, including the liver. The hydrostatic pressure forces pDNA into the liver cells before being mixed with blood (Liu *et al.*, 1999). On the other hand, a receptor-mediated process is also hypothesized for the *in vivo* uptake of naked pDNA by hepatocytes (Budker *et al.*, 2000). The gene expression level obtained by this approach is much better than those with other approaches using naked pDNA or nonviral vectors.

Such huge differences in gene expression by naked pDNA between a normal (small volume, slow) injection and a large volume injection at a high velocity would in part be explained by differences in the pharmacokinetic profile of the injected pDNA. Kobayashi *et al.* (2001) studied the biodistribution of naked ^{32}P-pDNA in mice following normal or large volume injection. In both cases, radioactivity accumulated largely in the liver. However, the hepatic uptake of ^{32}P-pDNA following normal injection was saturable and was inhibited by pDNA itself, calf thymus DNA, polyinosinic acid (poly I), and chondroitin sulfate. On the other hand, the uptake following the large volume injection was not inhibited by the administration of poly I, dextran sulfate, and heparin. In addition, proteins such as bovine serum albumin (BSA), which is hardly taken up by tissues, including the liver, in a normal condition, are also taken up, by the liver by this large volume injection. These findings suggest that the uptake of pDNA following the large volume injection is a nonspecific process, while its uptake following the conventional injection would be mediated by scavenger receptor-like mechanism(s) (Kawabata *et al.*, 1995; Takakura *et al.*, 1999).

Oligonucleotides

Compared with pDNA, oligonucleotides are very small in size, and they can be susceptible to glomerular filtration after systemic administration. Contrary to the invariable properties of pDNA, the physicochemical properties of oligonucleotides, such as the molecular weight, hydrophilicity/hydrophobicity and electrical charge, vary depending on the nature of the linkage between nucleotides. Such variations are reflected in the pharmacokinetic profile of oligonucleotides.

Due to the small size (smaller than 10 kD in most cases), oligonucleotide can be easily filtered at the kidney. In addition, its property of plasma protein binding will greatly alter the biodistribution of oligonucleotides. Compared with natural phosphodiester oligonucleotide, the phosphorothioate one has a much higher affinity with serum proteins, including albumin. A 10-mer phosphodiester oligonucleotide showed little binding with bovine serum albumin (BSA) in buffer solution and, when administered into the perfused rat kidney, it was efficiently excreted into urine (Sawai *et al.*, 1995). On the other hand, its phosphorothioate showed a high binding ability to BSA, resulting in reduced filtration at the kidney.

When injected intravenously in mice, a 10-mer phosphordiester oligonucleotide modified with ^{3}H-biotin at the 5′-end and with methoxyethylamine at the 3′-end was very quickly metabolized within the body; more than 90% of radioactivity in plasma was degradation product at two minutes after injection (Miyao *et al.*, 1995). Mahato *et al.* (1997) examined the biodistribution of ^{35}S-labeled 20-mer phosphordiester and phosphorothioate oligonucleotides following systemic injection in mice. Both types of oligonucleotides were eliminated rapidly from the circulation,

but the tissue distribution was different. A pharmacokinetic analysis based on the clearance concept showed the phosphordiester oligonucleotide had relatively large uptake clearances for all internal organs tested and large urinary excretion clearance, while the phosphorothioate possessed small urinary clearance, reflecting its high ability of plasma protein binding.

Due to the stability and resistance to endonuclease and exonuclease degradation (Uhlmann and Peyman, 1990), phosphorothioate linkage becomes the first choice of antisense oligonucleotides. Intense accumulation of phosphorothioate oligonucleotides in the liver and kidney is a universal distribution characteristic observed in many studies (Agrawal *et al.*, 1991, 1995; Sands *et al.*, 1994; Phillips *et al.*, 1997; Peng *et al.*, 2001), irrespective of the length of oligonucleotide between 20 and 25-mer. Recently, Peng *et al.* (2001) analyzed the biodistribution of ISIS 1082, a 21-mer phosphorothioate oligonucleotide in rat, based on a physiologically based pharmacokinetic model. They also used a model, similar to the model shown in Figure 19.1, in which all tissues were represented by a two-compartment model, comprising a blood compartment and an extravascular tissue compartment, with a permeability barrier at the capillary wall membrane. They found that the equilibrium tissue/plasma partition coefficient (*Kp*) of oligonucleotides exceeds one for all internal tissues, despite its relatively large size (about 6 kD). This *Kp* could be due to the high ability of phosphorothioate oligonucleotides for binding to tissue matrix. However, they could not find any correlation between the estimated permeability-surface area product in a tissue and its corresponding *Kp* value. This lack of correlation might be explained by the fact that permeability is a dynamic parameter, whereas *Kp* reflects a value at steady state.

Nonviral vector complex

The addition of cationic liposomes or polymers to a gene drug decreases its negative charge and facilitates its interaction with the cell membrane, so various vectors have been developed and used for *in vivo* delivery of a gene drug. One major group of these vectors consists of cationic lipids or liposomes. After systemic injection, cationic lipid/pDNA complex resulted in gene expression in vascular endothelial cells (Zhu *et al.*, 1993; Liu *et al.*, 1997), especially the cells in the lung, the first tissue a pDNA complex encounters following injection.

We examined the biodistribution of cationic liposomes/pDNA complex following intravenous injection in mice and pharmacokinetically analyzed the data based on the clearance concept (Mahato *et al.*, 1995a, 1997). These analyses showed that the pharmacokinetics of ^{32}P-pDNA complexes depend on their mixing (charge) ratio, the type of cationic and helper lipids (Mahato *et al.*, 1998). When analyzed using radioactivity counting following the injection of the complex prepared with ^{32}P-pDNA, the tissue uptake clearance per g tissue (mL/h/g tissue) was large for the lung, liver and spleen, though gene expression was not correlated with this uptake characteristic; no gene expression was detected in the liver in any formulation (Mahato *et al.*, 1995b).

The preferential gene transfer in the lung following intravenous injection of cationic lipid/pDNA complex would result from complicated events occurring in

the body. Positively charged liposomes/pDNA complexes bind to serum components, such as albumin, heparin, lipoprotein, or specific opsonins. This interaction is dependent on the net charge density and surface morphology of lipid/pDNA complexes (Mahato *et al.*, 1998). The biodistribution of pDNA after intravenous injection was dependent on the charge ratio of cationic liposomes/pDNA; the amount in the lung decreased by ten-fold, but that in the liver increased when the charge ratio decreased from 3:1 to 0.5:1 (+/−) (Mahato *et al.*, 1998). Sakurai *et al.* (2001) studied the tissue distribution of cationic liposomes/pDNA complexes in mice following intravenous injection, with or without preincubation of the complexes with serum or red blood cells (RBC). When a formulation contains dioleoylphosphatidylethanolamine (DOPE) as a helper lipid, cationic liposomes/pDNA complexes pre-incubated with RBC resulted in embolization in the lung, whereas a formulation with cholesterol instead of DOPE did not. Cationic liposomes/pDNA complexes induced fusion and aggregation of erythrocytes *in vitro* when DOPE was added as a helper lipid (Sakurai *et al.*, 2001). These differences in the interaction characteristics of pDNA complexes with blood components partially explain the differences in the biodistribution following intravenous injection of various complex formulations (Mahato *et al.*, 1998).

Cationic polymer is also frequently examined to increase the potential of a gene drug. Large molecular weight cationic polymers can condense pDNA more efficiently than cationic liposomes. They include: poly-L-lysine (PLL), poly-L-ornithine, polyethyleneimine (PEI), chitosan, starburst dendrimer and various novel synthetic polymers. These polymers can enhance the cellular uptake of pDNA by nonspecific adsorptive endocytosis.

Biodistribution of cationic polymer/pDNA complexes is more easily controlled than that of cationic lipid/pDNA complexes, therefore, active targeting to a specific population of cells in the body has been attempted since 1988 (Wu and Wu, 1988). Polymers such as PLL and PEI have been covalently modified with targeting ligand, which include asialoglycoproteins (Wu and Wu, 1988), carbohydrates (Perales *et al.*, 1994), transferrin (Kircheis *et al.*, 1999), and antibody (Li *et al.*, 2000). However, the pharmacokinetics of cationic polymer/pDNA complexes used in these studies was hardly examined. We determined the biodistribution profiles of galactosylated PLL (Gal-PLL)/pDNA complexes following intravenous injection in mice (Nishikawa *et al.*, 1998). As mentioned above, naked pDNA is rapidly taken up by the liver. Cell fractionation and confocal imaging of fluorescein-labeled pDNA following intravenous injection in mice has shown that pDNA is mainly taken up by sinusoidal cells such as Kupffer cells and endothelial cells (Kawabata *et al.*, 1995; Kobayashi *et al.*, 2001). Since the uptake by these cells seems to be mediated by the strong negative charge of pDNA (Takagi *et al.*, 1998b; Takakura *et al.*, 1999) and its clearance is very large (about 80 mL/h, Figure 19.3), it is important to mask the negative charge of pDNA for controlling its biodistribution. The pharmacokinetics of the DNA complexes are determined by not only a ligand but also by the overall physicochemical characteristics of the complexes (as summarized in Figures 19.2 and 19.3). After the intravenous injection of Gal-PLL/^{32}P-pDNA complexes, the hepatic uptake clearance was much greater than any of the other tissue uptake clearances. However, the physicochemical properties of Gal-PLL used for the complexation greatly affected the pharmacokinetics of the pDNA complex. Figure 19.4 shows the total body and hepatic uptake clearances of

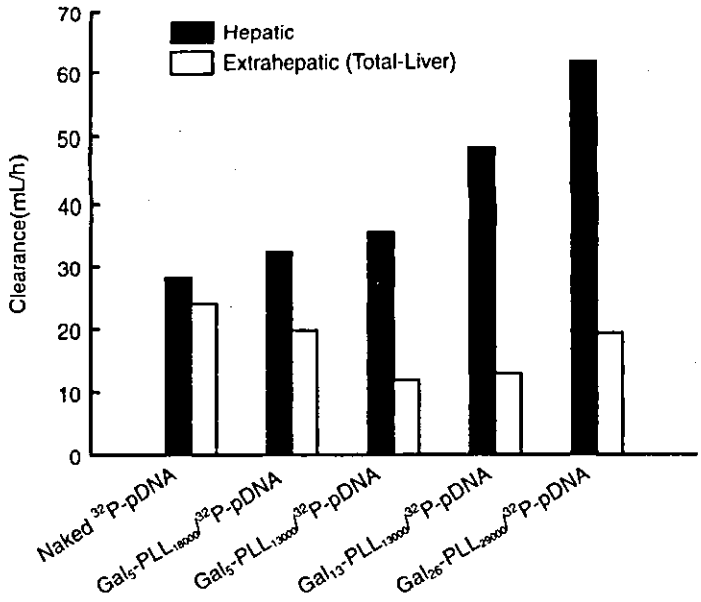

Figure 19.4 Hepatic and extrahepatic clearances of ^{32}P-pDNA and its complexes with varying Gal-PLL following intravenous injection in mice. The hepatic clearance was calculated by dividing the amount of radioactivity in the liver at an appropriate time point by the AUC up to the same time. Extrahepatic clearance represents the difference in the total-body and hepatic clearances. The numbers in the subscript represent the number of galactose (Gal-) and the molecular weight of PLL.

various Gal-PLL/^{32}P-pDNA complexes. The clearance values demonstrate that the complexes with larger Gal-PLL (13 or 29 kD in the molecular weight of PLL) have larger hepatic (target) clearance than ones with small Gal-PLL (1.8 kD), which failed to deliver efficiently pDNA to hepatocytes, probably due to the complex dissociation before reaching the target.

The figure also shows the importance of the number of galactose residues on PLL for the pDNA delivery using Gal-PLL (compare the results of complexes with Gal$_5$-PLL$_{13000}$ and Gal$_{13}$-PLL$_{13000}$), as clearly demonstrated in the studies of galactosylated or mannosylated proteins (Nishikawa *et al.*, 1995; Opanasopit *et al.*, 2001). Gene expression following intravenous injection corresponded to these pharmacokinetic profiles of Gal-PLL/pDNA complexes (Nishikawa *et al.*, 1998). The pDNA complexes with cationic carriers would be internalized by cells via endocytosis, resulting in lysosomal degradation. This intracellular pathway greatly limits the efficiency of gene transfer by this approach. In addition to the control of *in vivo* pharmacokinetics by using carrier molecules like Gal-PLL, the control of intracellular sorting of pDNA can be a good approach to increase gene transfer at the target. Wagner *et al.* (1992) demonstrated the increased gene expression in cultured cells by the addition of fusogenic peptides, derived from influenza virus hemmaglutinin subunit HA-2, to pDNA complexes. We attached fusogenic peptide

to a hepatocyte-targetable polymer and obtained an improved gene expression in the liver, indicating that the peptide also works in whole animals to, at least partially, avoid the intracellular degradation (Nishikawa *et al.*, 2000).

Similar to pDNA, the pharmacokinetics of oligonucleotides can be controlled by using delivery systems. Mahato *et al.* (1997) studied the pharmacokinetic characteristics of antisense oligonucleotides complexed with Gal-PLL or mannosylated PLL (Man-PLL), whose mannose residues can be recognized by mannose receptors on macrophages and liver sinusoidal endothelial cells (Shlesinger *et al.*, 1978). Complex formation of phosphodiester or phosphorothioate oligonucleotides with either of these modified PLL greatly reduced the urinary excretion clearance by increasing the effective size of the oligonucleotide, about 100–160 nm in diameter when measured by laser light scattering (Mahato *et al.*, 1997). Increased hepatic uptake clearances were obtained by the complex formation with Gal- or Man-PLL, but the delivery efficiency was not so outstanding for oligonucleotides as observed in pDNA delivery. Such differences suggest that the molecular weight of both components of gene drugs/cationic carrier complexes determine the stability of the complex, which is a major determinant of its pharmacokinetics.

LOCAL PHARMACOKINETICS

Isolated perfused tissue is a good system with which to analyze the pharmacokinetic events occurring in a specific tissue of interest following administration into the artery or by direct injection into the tissues. When isolated from the other parts of the body and perfused without recirculation (single-pass, one-loop system), the tissue can be pharmacokinetically treated as a physiological one-organ model. In this model, the circulatory system is observed as a black box under linear disposition conditions and the disposition function of a drug is reflected in the output response to an input. The output response is obtained by way of the time course of the outflow concentration and can usually be regarded as a statistical distribution curve, similar to that observed in chromatography. Statistical moment analysis was applied to a single-pass local perfusion system (Kakutani *et al.*, 1985). These pharmacokinetic approaches have been applied to analyze the disposition characteristics of various drugs in the liver (Nishida *et al.*, 1989, 1990, 1991a, 1991b, 1992; Takakura *et al.*, 1996; Yoshida *et al.*, 1996; Takagi *et al.*, 1998b; Ogawara *et al.*, 1998, 1999), kidney (Mihara *et al.*, 1993a, 1993b, 1994; Sawai *et al.*, 1995, 1996; Takagi *et al.*, 1997) and tumor (Ohkouchi *et al.*, 1990; Imoto *et al.*, 1992; Saikawa *et al.*, 1996; Nomura *et al.*, 1997, 1998a, 1998b; Nakajima *et al.*, 2000). Here, we summarize the pharmacokinetics of gene drugs in these isolated tissues.

Isolated perfused liver

In the isolated perfused liver experiments, buffers containing no erythrocytes or serum proteins are used to examine the direct interaction of a gene drug with tissues and to avoid the interaction of a gene drug with blood components and possible contamination of nucleases.

Bolus injection

The statistical moment parameters for the outflow pattern of a bolusly administered drug from the portal vein are defined as follows:

$$AUC = \int_0^\infty C dt \tag{9}$$

$$\bar{t} = \int_0^\infty \frac{tC}{AUC} dt \tag{10}$$

where t (sec) is the time and C (% of dose/mL) is the concentration of compound. AUC (% of dose sec/mL) and $t(bar)$ (sec) denote the area under the concentration-time curve and mean transit time of the drug through the liver, respectively. The moments defined by Eq (9) and (10) can be calculated by numerical integration using a linear trapezoidal formula and extrapolation to infinite time based on a monoexponential equation. The $t(bar)$ values are corrected for the lag time of the catheter.

The hepatic disposition parameters of a drug, representing reversible and irreversible processes, are calculated using the following equations:

$$F = AUC \cdot Q \tag{11}$$

$$V = \frac{Q \cdot \bar{t}}{F} \tag{12}$$

$$E = 1 - F \tag{13}$$

$$t_{cor} = \frac{\bar{t}}{F} \tag{14}$$

$$k_{el} = \frac{E}{\bar{t}} \tag{15}$$

$$CL_{int} = k_{el} \cdot V \tag{16}$$

where V (mL) is the apparent retention volume, which reflects reversible interaction of drug with the tissue; t_{cor} (sec) is the corrected mean transit time, E (%) is the extraction ratio, k_{el} (min^{-1}) is the first order irreversible elimination rate constant, CL_{int} (mL/min/g tissue) is the intrinsic clearance, and Q (mL/min) is the perfusion rate. These parameters can be divided into three groups: parameters representing reversible interaction (V, t_{cor}), irreversible uptake (E, F, k_{el}), and both (CL_{int}) (Nishida *et al.*, 1990).

The pharmacokinetic analysis of the outflow dilution curve of the concentration of radioactivity following bolus injection of ^{32}P-pDNA and ^{32}S-oligonucleotides with different linkages translates the distribution data to the quantitative parameters (Table 19.1). The extraction (E) of gene drugs in the perfused liver was relatively high for ^{32}P-pDNA (46% at 1.33 μg/liver), ^{32}P-PS$_3$ (33% at 3 μg/liver) and ^{32}P-PS (36% at 3 μg/liver), but low for ^{32}P-PO (15% at 3 μg/liver). These results correspond to the *in vivo* distribution data following systemic administration into mice (Kawabata *et al.*, 1995; Miyao *et al.*, 1995). If a drug does not reversibly