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Theoretical considerations involving the pharmacokinetics of plasmid DNA

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Abstract

Success of in vivo gene therapy relies on the development of gene delivery technologies, by which a well-controlled transgene expression is achieved as far as the spatial and temporal profile of the expression is concerned. Because transgene expression only occurs in cells that are transduced with the gene administered, the tissue distribution of genes is an important factor determining the efficacy of in vivo gene transfer. Plasmid DNA is the simplest vector and its administration in naked or complexed form results in significant transgene expression in various organs. The route of administration, the use of cationic vectors and the administration technique greatly affects the tissue distribution of plasmid DNA and the subsequent transgene expression. Therefore, a clear understanding of the tissue distribution of naked and complexed plasmid DNA is a prerequisite for strategies for developing effective in vivo gene transfer methods. Pharmacokinetics translates the tissue distribution properties of plasmid DNA into quantitative parameters, which can be compared with parameters obtained under different conditions, or with physiological parameters such as blood flow rate. Here we discuss the pharmacokinetic evaluation of the tissue distribution characteristics of plasmid DNA, in the free and complexed forms.
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Keywords: Gene transfer; Tissue distribution; Clearance; Receptor-mediated endocytosis

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1. Introduction

In vivo gene replacement therapy requires the development of gene delivery technologies, by which a well-controlled transgene expression is achieved as far as the spatial and temporal profile of the expression is concerned. Although genes can be easily introduced to cells ex vivo by means of viruses, cationic liposomes (lipids) or electric pulses, their administration directly to patients represents an ideal methodology for the treatment of a variety of diseases. Following in vivo administration, however, genes encounter various hurdles that need to be overcome for a successful in vivo gene therapy (Fig. 1).

Transgene expression only occurs in cells that are transduced with the gene administered. Therefore, the tissue distribution of genes is an important factor determining the efficacy of in vivo gene transfer. Generally speaking, the tissue distribution of an externally administered compound is determined by its interaction with the body, and such interaction is regulated by the physicochemical and biological properties of the compound and the anatomical and physiological properties of the body. Therefore, drug targeting depends on optimizing the properties of the drug delivery system [1,2], or by altering the properties of the body. One example of the latter is the osmotic opening of the blood–brain barrier [3]. Theoretically, changing the route of administration, for example intraarterial injection to the target organ instead of intravenous injection, is also a promising approach for improving the targeting efficiency of a drug [4]. Similar strategies could be applied to genes for delivering them to target cells. However, unlike most conventional drugs, genes need to find a way into the intracellular space where they can then exert their effects.

Since Wolff et al. [5] reported that transgene products can be obtained in skeletal muscle by a simple intramuscular injection of naked plasmid DNA, it is now widely accepted that plasmid DNA is a promising nonviral vector for in vivo gene transfer. Even for systemic administration, naked plasmid DNA can produce high levels of transgene product when it is rapidly injected into the systemic circulation in a large-volume solution [6]. However, a conventional intravenous injection of plasmid DNA results in undetectable transgene expression in major organs [7,8]. Altered tissue and intracellular distribution of plasmid DNA would explain such differences in the final outcome of transgene expression (see the review by Kobayashi et al. [9] in this issue). To improve the delivery and cellular uptake of plasmid DNA after in vivo administration, a number of cationic delivery systems have been developed. Plasmid DNA forms electrostatic complexes with these cationic vectors and, within the body, such complexes interact with various components and show complicated distribution profiles.

A clear understanding of the tissue distribution of plasmid DNA and its complexes with nonviral vectors is a prerequisite for a strategy for developing novel in vivo gene transfer methods. Pharmacokinetics translates the tissue distribution properties of plasmid DNA into quantitative parameters, which can be compared with parameters obtained under different conditions, or with physiological parameters such as blood flow rate and the rate of fluid-phase endocytosis. Here, we discuss the pharmacokinetics of the tissue distribution characteristics of plasmid DNA, which is systemically

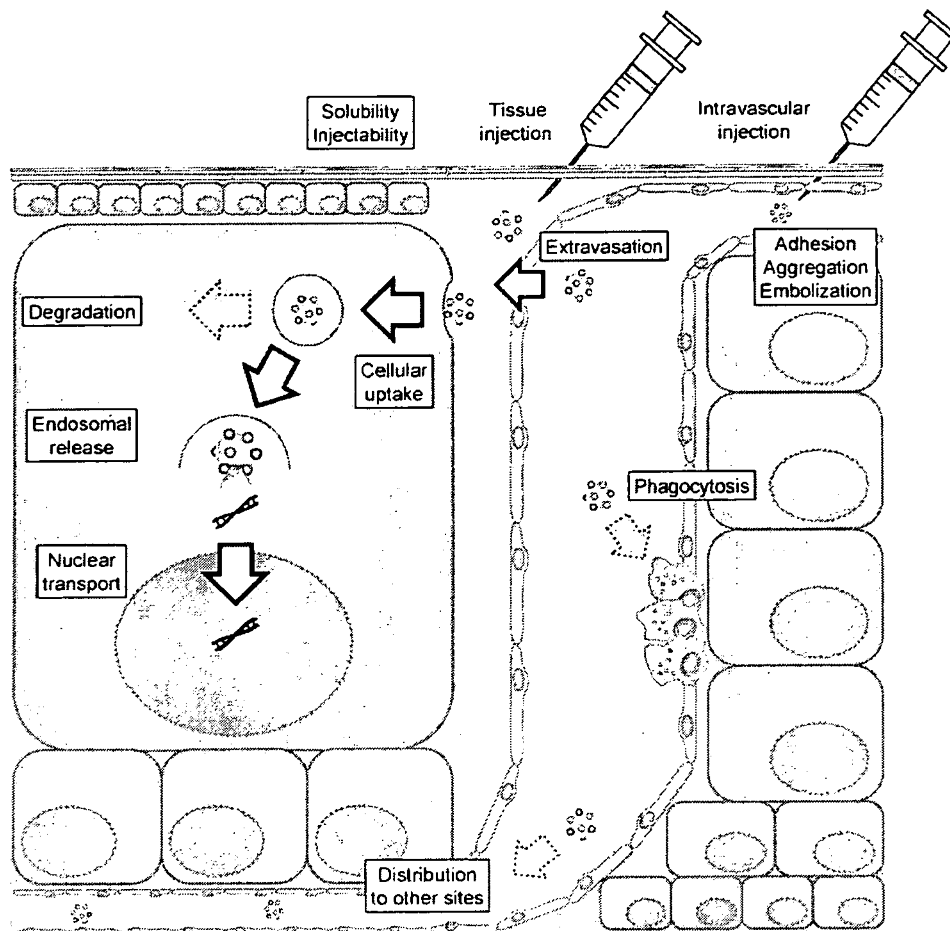

Fig. 1. Hurdles in gene delivery following in vivo administration. The vector system should be soluble and injectable, avoid adhesion (non-specific binding) to tissues, aggregation, embolization, and phagocytosis, extravasate, find a way to the inside of target cell, escape from endosomal/lysosomal degradation, and be transported into the nucleus. When any vector or delivery system is used for the delivery of plasmid DNA, the DNA should be released prior to the nuclear entry.

administered in the free and complexed forms. Because plasmid DNA possesses several features common to other macromolecular compounds in terms of tissue distribution, the pharmacokinetic characteristics of macromolecules are first summarized. Cell-specific delivery approaches that have been applied to targeted gene delivery are also evaluated in pharmacokinetic terms.

2. Pharmacokinetic analysis of tissue distribution characteristics of macromolecules

Although, if not protected, plasmid DNA is easily degraded by nucleases following administration, fractions maintaining the structure could behave as macromolecules in vivo. Therefore, it is important to summarize the factors determining the tissue distribution characteristics of macromolecules. Pharmacokinetic analysis based on the concept of clearance is used to quantitatively evaluate the determinants affecting the tissue distribution of macromolecules.

2.1. Theoretical background of the analysis

2.1.1. Uptake by target

The tissue distribution of a macromolecule via the circulation can be pharmacokinetically analyzed based on the concept of clearance. Tissue uptake

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

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

where X_i (μg) represents an amount of the macromolecule in tissue i after administration, C_p (μg/ml) is its concentration in the plasma, $CL_{app,i}$ (ml/h) expresses the apparent tissue uptake clearance from the plasma to tissue i, and $k_{efflux,i}$ (h^{-1}) represents the efflux rate from tissue i.

An assumption of negligible efflux from the tissue ($k_{efflux,i}$=0) makes it easier to pharmacokinetically analyze the distribution process of macromolecules. Macromolecular drugs, such as proteins and plasmid DNA, are labile to undergo degradation before and after cellular uptake. When traced with radioisotopes, radioactive metabolites are sometimes rapidly released from the cells that have taken up the radiolabeled compound. This is the case when galactosylated bovine serum albumin (Gal-BSA), a macromolecular ligand for the asialoglycoprotein receptors on hepatocytes [10,11], undergoes direct ^{125}I radiolabeling on the tyrosine residues. Fig. 2A shows the radioactivity in mouse liver after intravenous injection of Gal-BSA labeled with ^{125}I or ^{111}In [12]. Upon administration, Gal-BSA is rapidly taken up by the liver via the asialoglycoprotein receptor-mediated endocytosis. The endocytosed material is degraded within the lysosomes [13,14], which leads to the release of radioactive metabolites from the liver. This release makes it difficult to estimate the uptake clearance by the liver. On the other hand, little radioactivity was released from the liver during the experimental period when Gal-BSA was labeled with ^{111}In using diethylenetriaminepentaacetic dianhydride as a chelating agent (Fig. 2A). The physicochemical properties of radioactive metabolites will explain such differences [15], and 'residualizing' radiolabels which support the assumption that there is little efflux from tissues after uptake have been investigated for radioiodine [16–18] and metallic chelates [19–21]. These results indicate that if a macromolecule is labeled by a proper method, the efflux process in the pharmacokinetic analysis can be ignored even though the compound itself undergoes intracellular degradation. Based on these considerations, we recently developed a residualizing radiolabel for plasmid DNA [22]. Compared with its ^{32}P-labeled counterpart, ^{111}In-labeled naked plasmid DNA showed a prolonged retention of radioactivity in the liver after intravenous injection in mice (Fig. 2B).

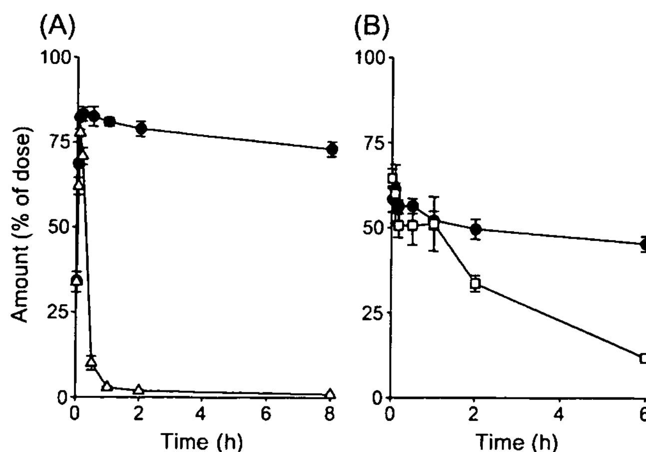

Fig. 2. Time courses of radioactivity in the liver after intravenous injection of (A) galactosylated bovine serum albumin (Gal-BSA) or (B) plasmid DNA in mice after intravenous injection. Data are expressed as the mean±S.D. of three mice. (A) ●, ^{111}In-Gal-BSA; △, ^{125}I-Gal-BSA. (B) ●, ^{111}In-plasmid DNA; □, ^{32}P-plasmid DNA.

Under the assumption of negligible efflux from the tissue, Eq. (1) is simplified to:

$$\frac{dX_i}{dt} = CL_{app,i}C_p \quad (2)$$

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

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

where AUC_p (μg h/ml) is the area under the plasma concentration–time curve of the macromolecule. Its elimination profile from the plasma can be expressed as a function of one or more exponentials in many cases. Then, the AUC_p values at any time point can be calculated by fitting an equation to the experimental data using a least-squares method. According to Eq. (3), $CL_{app,i}$ is calculated from the slope of the plot of the amounts in the tissue (X_i) against AUC_p. Table 1 summarizes the AUC and clearances of several model macromolecules in mice after intravenous injection; these parameters clearly indicate that the tissue distribution characteristics of macromolecules are dependent on parameters, such as the molecular weight and electric charge as discussed below.

Tissue uptake clearances are useful parameters for characterizing the tissue distribution properties of a macromolecule because they are independent on its concentration in the plasma. However, the tissue uptake process sometimes depends on its concentration in plasma and follows non-linear kinetics. Then, the calculated $CL_{app,i}$ would represent an average value of its time-dependent clearances for the overall experimental period [10].

$CL_{app,i}$ is a hybrid parameter of the plasma flow rate (Q, ml/h) to the tissue and the intrinsic uptake clearance ($CL_{int,i}$) by the tissue. Therefore, it can be expressed as:

$$CL_{app,i} = \frac{QCL_{int,i}}{Q + CL_{int,i}} \quad (4)$$

This equation clearly demonstrates the limitation of gene delivery to tissues with a low blood flow rate; if $CL_{int,i}$ is successfully increased by any means, $CL_{app,i}$ approaches to Q. Table 2 summarizes the blood flow rate to various tissues in human [23]. Compared with the rate normalized for tissue weight, the blood flow to the kidney, liver, and brain is much higher than that to the skin, muscle, and fat. Therefore, even although skeletal muscle is an important target for in vivo gene transfer, it is difficult to deliver plasmid DNA to the

Table 1
AUC and clearances of model macromolecules in mice after intravenous injection

Compound	Molecular weight (kDa)	Charge, ligand	Dose (mg/kg)	AUC (%dose h/ml)	CL_{total}	$CL_{app,liver}$	CL_{urine}
^{111}In-plasmid DNA	~4000	Strongly anionic	0.5	2.8	36.0	17.8	–
^{111}In-Suc_{40}-BSA	70	Strongly anionic	0.1	1.5	64.9	48.0	0.17
^{14}C-dextran sulfate	8	Strongly anionic	1	4.0	25.3	2.47	12.3
^{3}H-oligo DNA	3	Strongly anionic	1	1.3	75.0	11.1	17.0
^{111}In-BSA	67	Anionic	1	1430	0.07	0.02	0.01
^{14}C-CM-dextran	70	Anionic	100	1010	0.10	0.01	0.07
^{14}C-dextran	70	Neutral	100	146	0.69	0.24	0.26
^{14}C-dextran	10	Neutral	100	6.6	15.2	0.24	12.8
^{14}C-DEAE-dextran	70	Cationic	100	51	1.96	0.36	1.06
^{111}In-PLL	60	Cationic	1	0.6	159	66.1	0.32
^{111}In-Cat-BSA	70	Cationic	1	2.8	35.5	24.8	0.40
^{111}In-Gal_{36}-BSA	70	Anionic, Gal	0.1	1.2	85.0	77.3	0.16
^{111}In-Man_{16}-BSA	70	Anionic, Man	0.1	5.0	20.0	14.3	n.d.
^{111}In-Gal_{28}-PLL	60	Cationic, Gal	1	0.6	175	97.1	0.19

BSA, bovine serum albumin; Suc-, succinylated; CM-dextran, carboxymethyl-dextran; DEAE-dextran, diethylaminoethyl-dextran; PLL, poly-L-lysine; Cat-, cationized; Gal-, galactosylated; Man-, mannosylated. n.d., not determined. These data are cited from our previous studies [2,11,22,36,40,80].

Table 2
Human tissue volume and blood flow rate to tissues [23]

Tissue	Male			Female		
	Volume (ml)	Flow (ml/min)	Perfusion rate (ml/min/ml)	Volume (ml)	Flow (ml/min)	Perfusion rate (ml/min/ml)
Brain	1400	720	0.51	1200	624	0.52
Kidneys	310	1140	3.68	275	884	3.22
Liver	1800	(1500)	(0.84)	1400	(1404)	(1.00)
Arterial		390	0.22		338	0.24
Portal		1110	0.62		1066	0.76
Muscle	30000	1020	0.03	18000	624	0.03
Fat	12500	300	0.02	17500	442	0.03
Subcutaneous	7500			13000		
Other	5000			4500		
Heart: without blood	330	240	0.73	270	260	0.96
Skin	(2600)	300	0.12	(1790)	260	0.15
Epidermis	100			90		
Dermis	2500			1700		
Splanchnic	(1480)	(1140)	–	(1365)	(1092)	–
Pancreas	100	60	0.60	85	52	0.61
Spleen	180	180	1.00	150	156	1.04
GI tract	(1200)	(900)	–	(1130)	(884)	–
Stomach	150	60	0.32	140	52	0.31
Intestine	(1010)	(840)	–	(960)	(832)	–
Small	640	600	0.94	600	572	0.95
Large	370	240	0.65	360	260	0.72
Skeleton	10500	300	0.03	8500	260	0.03
Remainder	4936	355	–	4291	328	–
Total	73000	6000	–	60000	5200	–

organ via the vascular route under a normal conditions. To overcome this limitation, improvement in administration techniques is needed. Efficient delivery of plasmid DNA to skeletal muscle or the diaphragm muscle has been successfully achieved by temporally restricting the distribution of plasmid DNA only to the target tissue [24–26].

2.1.2. Balance of the clearances of target and non-target tissues

The total body clearance (CL_{total}, ml/h) of a macromolecule can be calculated using AUC_p for infinite time ($AUC_{p,\infty}$) and the dose (D) as follows:

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

Because CL_{total} can be considered as the sum of CL_{target} (the uptake clearance of the target tissue), $CL_{non\text{-}target}$ and the degradation clearance within the systemic circulation (CL_{deg}), the fraction of the macromolecule delivered to the target (F_{target}) can be calculated as:

$$F_{target} = \frac{CL_{target}}{CL_{total}} = \frac{CL_{target}}{CL_{target} + CL_{non-target} + CL_{deg}} \tag{6}$$

Therefore, the potential of the targeted delivery of plasmid DNA can be quantitatively explained by the parameters of CL_{target}, $CL_{non\text{-}target}$, and CL_{deg}. Eq. (6) clearly indicates that an approach increasing the CL_{target} and/or reducing the $CL_{non\text{-}target}$ or CL_{deg} of plasmid DNA is suitable for achieving an efficient gene transfer at the target site.

2.2. Anatomical and physiological properties of the body

Macromolecules entering the systemic circulation distribute to tissues largely via the bloodstream. Therefore, as discussed above, the blood flow rate

determines the delivery rate of macromolecules to each tissue.

Macromolecules in the circulation have a direct access to the capillary endothelial cells as well as various circulating cells in the blood. These cells have the opportunity to take up macromolecules via specific or nonspecific interactions. The interaction of macromolecules with parenchymal cells in tissues can occur only when they have access through the endothelial lining. The structure of the blood capillary wall varies greatly depending on the organ. In addition, pathological states such as inflammation could change the structure. On the basis of the morphology and continuity of the endothelial layer and the basement membrane, the capillary endothelium can be divided into the continuous, fenestrated and discontinuous endothelium [27,28].

Tight junctions between endothelial cells and underlying uninterrupted basement membrane characterize the continuous endothelium, through which the passage of macromolecules is greatly restricted. This type of endothelium can be found in skeletal, cardiac, and smooth muscles, and in lung, skin, and subcutaneous tissues. Macromolecules of around 6 nm in diameter or more hardly interact with parenchymal cells in these tissues simply due to the barrier posed by the endothelium. Endothelial cells having fenestrae equipped with a diaphragm, an opening 40–80 nm in diameter, form the fenestrated endothelium in the intestinal mucosa, the endocrine and exocrine glands, and the glomerulus and peritubules of the kidney. However, the passage of macromolecules through this type of endothelium is limited by the presence of the basement membrane. A clear relationship between the molecular weight and glomerular filtration has been reported using dextrans [29] and other macromolecules [30]; macromolecules with a molecular weight of 40 kDa or more are scarcely excreted into the urine. Discontinuous or sinusoidal endothelium is found only in the liver, spleen, and bone marrow. These capillaries are characterized by endothelial gaps, intercellular junctions with a diameter up to 30–500 nm and with either no basement membrane (liver) or a discontinuous basement membrane (spleen and bone marrow). Therefore, parenchymal cells in these tissues can be accessed by macromolecules with relatively high molecular weight. Enhanced vascular permeability of macromolecules is also found in solid tumors [31].

2.3. Physicochemical properties of macromolecules

Depending on the anatomical and physiological properties of tissues, macromolecules are delivered to the vicinity of various cells in the body. Then, they interact with some of those cells depending on their physicochemical properties. The summation of such interactions determines the overall pharmacokinetics of each macromolecule. The physicochemical properties such as the size, charge, and specific structures recognized by any specific molecule on cell surface govern the interaction; this is the basis of designing a drug delivery system. Understanding the effects of the physicochemical properties of macromolecules on their tissue distribution allows us to theoretically design delivery systems not only for drugs but also for genes.

Because the passage through the endothelial cells is strictly regulated by size, the molecular weight of macromolecules greatly affects their tissue distribution. Macromolecules with a molecular weight of about 40 kDa or less pass through the renal glomerular capillary wall [29,30,32,33], resulting in a short plasma half-life. Oligonucleotides with a molecular weight less than the threshold undergo rapid glomerular filtration by the kidney [34,35]. Plasmid DNA, on the other hand, is too large to be filtered without degradation.

Another important characteristic of macromolecules is their electric charge. Because the cell surface is negatively charged, cationic molecules tend to electrostatically bind to it, resulting in greater tissue uptake. Although cationic macromolecules might bind to any type of cells in vivo, they are mainly delivered to the liver, kidney, or lung after intravenous administration. Cationic plasmid DNA complexes are generally trapped by the lung, which could be due to the direct interaction between cationic complexes and the endothelial cells in the lung, or to the embolization of aggregates of such complexes with blood components as discussed below. Neutral or weakly anionic macromolecules, such as polyethylene glycol and serum albumin, possess a very weak affinity for the cell surface, so they are cleared very slowly if the molecular size exceeds the threshold of the glomerular

filtration. A further reduction in the charge of the macromolecules, on the other hand, increases the affinity for cells having specific receptors that can be categorized as scavenger receptors [36].

2.4. Interaction with blood components

Although macromolecules distribute within the body depending on their physicochemical properties, the interaction with blood components is another factor regulating their tissue distribution [23].

Particulate carriers such as cationic liposomes that are used as nonviral vectors attract a number of serum proteins [37]. It has also been reported that the type and amount of serum proteins bound to the surface in part controls the tissue distribution of particulate carriers [38,39]. Some macromolecules can also interact with blood components. We have shown that mannan binding proteins in serum can bind to mannosylated macromolecules and alter their tissue distribution [40].

Blood cells also contribute to the interaction of cationic plasmid DNA complexes after intravenous injection. Sakurai et al. [41,42] reported that some plasmid DNA complexes with cationic liposomes bind to erythrocytes, form large aggregates and are captured by the lung capillaries.

2.5. Receptor-mediated uptake

Active targeting of genes will increase the therapeutic efficacy and reduced the potential side effects of in vivo gene therapy. Therefore, various approaches have been investigated to achieve targeting, such as the use of monoclonal antibodies or ligands for cell-surface receptors. Because of the cell-specificity of the expression, abundance, and other factors discussed above, receptors for transferrin, asialoglycoproteins, and mannose are the most common receptors that have been used in the targeted delivery of drugs and genes.

Theoretically, the use of a ligand for a receptor increases the uptake clearance of the target tissue, which leads to an increase in F_{target} in Eq. (6). This is the case when BSA, a model macromolecule, is modified with galactose or mannose [11,40]. Although receptor-mediated uptake is a capacity-limited process, the tissue distribution of galactosylated or mannosylated macromolecules can be analyzed using clearances calculated from Eq. (3); then the calculated value represents an average value of the time-dependent (concentration-dependent) clearances of ligands for the overall experimental period. In the case of the asialoglycoprotein receptor–ligand interaction, the binding affinity of the ligand is determined by the clustering and geometric organization of galactose, the number of galactose moieties, and so on. The effects of the number of galactose moieties on the macromolecules were clearly demonstrated by using poly-L-glutamic acid (PLGA) and BSA [11,43]. An increase in the number of galactose moieties increased the $CL_{app,liver}$, because hepatocytes in the liver only express asialoglycoprotein receptors. Because the receptor-mediated uptake of galactosy-

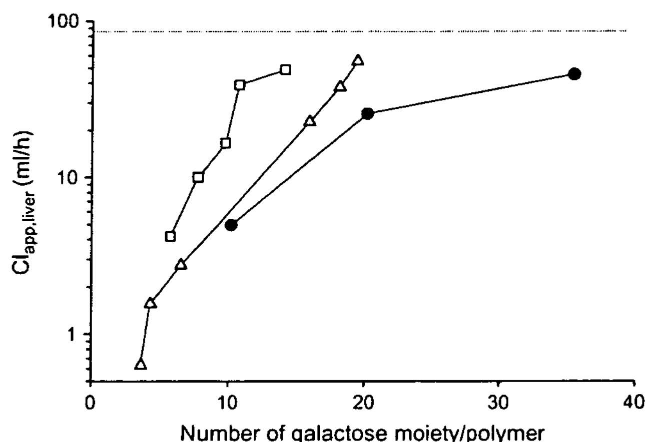

Fig. 3. Apparent hepatic uptake clearances ($CL_{app,liver}$) of galactosylated macromolecules after intravenous injection in mice at a dose of 1 mg/kg. The dashed line represents the plasma flow rate (Q) to the liver in mice. ●, ^{111}In-Gal-BSA; △, ^{111}In-Gal-PLGA, □, ^{111}In-Gal-SOD.

lated macromolecules is so efficient and fast, i.e., the $CL_{int,liver}$ is very large, the $CL_{app,liver}$ approaches to the hepatic plasma flow rate, Q, as the number of galactose moieties increases and the dose administered decreases (Fig. 3).

3. Pharmacokinetics of plasmid DNA following systemic, intravenous administration

Plasmid DNA is a macromolecule with a high molecular weight of about 2500 kDa or more and a strong anionic charge. As discussed above, these properties greatly limit the tissue distribution of plasmid DNA. Without degradation, the large size hinders the access of circulating plasmid DNA to cells except for the blood cells, endothelial cells, and parenchymal cells in the liver and spleen. Some of these cells take up and degrade plasmid DNA, resulting in little transgene expression following a simple intravenous injection of naked plasmid DNA. Cationic molecule-based nonviral vectors are used to increase the affinity of plasmid DNA for cells via electrostatic interaction but, as summarized above, they greatly alter the tissue distribution of plasmid DNA. Several ligands have been used to increase the selectivity of plasmid DNA delivery to target cells. For a very high transgene expression, naked plasmid DNA is injected into mice by the hydrodynamics-based procedure [6].

3.1. Naked DNA

The disappearance of naked plasmid DNA from the blood circulation has been examined in animals using several analytical methods. Houk et al. [44] described the plasma clearance of plasmid DNA after intravenous injection in rats. To detect the structural forms of the DNA, i.e., the supercoiled, open circular, and linear forms, they isolated plasmid DNA from whole blood samples by extraction. Then, the three plasmid DNA forms were separated by gel electrophoresis. They found that the supercoiled plasmid DNA is rapidly metabolized and cleared from the circulation, and is detectable in the bloodstream only after a very high dose of 2500 μg (about 7 mg/kg body weight). Separately, the amount of plasmid DNA in plasma after intravenous injection was also measured by quantitative polymerase chain reaction (PCR) [45]. At a dose of 50 μg to mice, a steady disappearance of the PCR products was observed in serum with a half-life of less than 1 min.

Although these analytical methods are effective in measuring plasmid DNA in solutions like serum, they are not appropriate for evaluating the tissue distribution. Cellular uptake is sometimes combined with a marked degradation of plasmid DNA, which could make it impossible to identify which cells or organs take up the DNA. The most promising alternative is the use of fluorescent- or radio-labeling of plasmid DNA. To date, a number of methods have been developed for studying not only the plasma clearance but also the tissue distribution of plasmid DNA. The most frequently used method for this purpose is ^{32}P-labeling by nick translation. When naked ^{32}P-plasmid DNA was injected as a bolus into the tail vein of mice at a dose of 1 mg/kg, radioactivity rapidly disappeared from plasma and a large fraction (60–70% of the injected dose) was recovered in the liver within 5 min of injection [7]. The pharmacokinetic analysis of the tissue distribution of ^{32}P-plasmid DNA revealed that the $CL_{app,liver}$ is close to the hepatic plasma flow rate, which accounts for a large fraction of the CL_{total}. In a different set of experiments, a rapid degradation in serum was also observed, and the degradation clearance was found to be large enough to digest the DNA with a half-life of less than 10 min. Nonviral vectors would protect the DNA from degradation in the circulation. Under these conditions, naked plasmid DNA resulted in no transgene expression in major organs [8].

Radiolabeling methods account for differences in the tissue distribution data of plasmid DNA. Although $CL_{app,tissue}$ is derived from Eq. (2) under the assumption of negligible efflux of compounds from tissues, the amount of ^{32}P-radioactivity in the liver decreases with time. In addition to ^{32}P-labeling, various methods have been developed to trace the tissue distribution of plasmid DNA. In this regard, radioiodination [46,47], metabolic labeling with ^{3}H-thymidine 5′-triphosphate [48], or ^{99m}Tc-label [49] have been used to follow the distribution of plasmid DNA in vivo. A number of fluorescence labeling methods are also available to trace the DNA [50–53]. However, the efflux rates of radioactive or fluorescent metabolites are generally too fast, so quantitative pharmacokinetics analyses are difficult to perform using these

labeled forms of plasmid DNA. To solve these problems, we recently developed a residualizing radiolabel for plasmid DNA using a hydrophilic metal chelate [22]. This label is designed so that the radioactive metabolites are locked in the cells that have taken up the labeled plasmid DNA. A good correlation was demonstrated between the amount of radioactivity delivered to the lung and the transgene expression in the organ.

3.2. Nonviral vector complex

The addition of cationic nonviral vector to plasmid DNA decreases its negative charge and facilitates its interaction with cell membranes, and so many such vectors have been developed and used for in vivo gene delivery.

3.2.1. Lipoplex

One major group of these vectors consists of cationic lipids or liposomes. After systemic injection, cationic lipid/plasmid DNA complex, or lipoplex, resulted in transgene expression in vascular endothelial cells [54,55], especially the cells in the lung, the first tissue the lipoplex encounters following intravenous injection. Cationic liposomes associate with plasmid DNA via an electrostatic interaction, which results in the formation of a complex [56]. The driving force for the lipoplex to introduce genes into cells is its electrostatic binding to negatively charged cellular membranes followed by endocytotic uptake. Some studies have shown that co-lipids, so-called helper lipids, in cationic liposomes are important determinants of the transfection efficiency. Intravenous injection of lipoplex can lead to a significant in vivo transfection activity in the lung when cholesterol is used as a helper lipid [55,57], although these lipoplex formulations produce less transfection in vitro than lipoplexes containing dioleoylphosphatidylethanolamine (DOPE). To compensate for the lack of cell-specificity in the electrostatic interactions of lipoplexes, ligands can be introduced into cationic liposomes. Sugars are the ligands that have been most extensively investigated so far [58–61].

We examined the tissue distribution of lipoplexes after intravenous injection in mice and carried out a pharmacokinetic analysis of the data based on the clearance concept [8,62], although the first-pass entrapment of lipoplexes by the lung makes it difficult to perform a pharmacokinetic analysis of their tissue distribution. These analyses showed that the pharmacokinetics of ^{32}P-plasmid DNA complexes depends on their mixing (charge) ratio, the type of cationic and helper lipids. When analyzed using radioactivity counting, the tissue uptake clearance per g tissue (ml/h/g tissue) was high for the lung, liver and spleen. The anatomical and physiological characteristics of these organs determine the enhanced delivery of the lipoplexes. The lung is the first organ that the lipoplexes encounter after intravenous injection, and the liver and spleen possess macrophages that take them up efficiently as well as the discontinuous endothelium that enables the lipoplexes to be taken up by parenchymal cells. However, the transgene expression was not correlated with such uptake characteristics, and little transgene expression was detected in the liver when conventional lipoplexes were intravenously injected [8]. The preferential gene transfer in the lung following intravenous injection of lipoplexes would result from complicated events occurring in the body, including the interaction with blood components. Sakurai et al. [41,42] studied the tissue distribution of lipoplexes in mice following intravenous injection, with or without preincubation of the complex with serum or red blood cells (RBC). When a formulation contained DOPE as a helper lipid, a lipoplex preincubated with RBC resulted in embolization in the lung, whereas a formulation with cholesterol instead of DOPE did not. These differences in the interaction characteristics of the plasmid DNA complex with blood components in part explain the differences in the tissue distribution following intravenous injection of various complex formulations [62]. Because the size of the lipoplex is a key factor determining the tissue distribution as well as the cellular uptake, a reduction in size would help increase the transfection efficiency by lipoplexes. Recently, Dauty et al. [63] succeeded in formulating plasmid DNA into stable nanometric particles with a diameter of less than 40 nm by synthesizing a dimerizable cationic detergent.

3.2.2. Polyplex

Cationic polymer/plasmid DNA complex, or polyplex, is another class of candidate that has been found to increase the transgene expression by plasmid DNA.

Large molecular weight-cationic polymers can condense plasmid DNA more efficiently than cationic liposomes, and this would be beneficial in controlling the tissue distribution of plasmid DNA complexes. These include: poly-L-lysine (PLL), poly-L-ornithine, polyethyleneimine (PEI), chitosan, starburst dendrimer and other novel synthetic polymers. These polymers can enhance the cellular uptake of plasmid DNA by nonspecific adsorptive endocytosis as cationic lipids do.

The tissue distribution of polyplex is more easily controlled than that of lipoplex, because there is less interaction between the cationic polymers and serum components. Therefore, active targeting to a specific population of cells in the body was attempted as early as 1988 [64]. Polymers such as PLL and PEI have been covalently modified with targeting ligand, and these include asialoglycoproteins [64], carbohydrates [65], transferrin [66], folate [67], and antibody [68]. However, the pharmacokinetics of the polyplexes used in these studies received little attention. We carried out a pharmacokinetic analysis of the tissue distribution of galactosylated PLL (Gal-PLL)/plasmid DNA complex following intravenous injection in mice [69]. As mentioned above, naked plasmid DNA is rapidly taken up by the liver. Cell fractionation and confocal imaging of fluorescein-labeled plasmid DNA following intravenous injection in mice showed that plasmid DNA is mainly taken up by sinusoidal cells such as Kupffer cells and endothelial cells [7,70]. Because the uptake by these cells seems to be mediated by the strong negative charge of plasmid DNA [71,72] and its clearance is very large, it is very important to mask the negative charge of plasmid DNA in order to control its tissue distribution. After intravenous injection of Gal-PLL/^{32}P-plasmid DNA complex, the hepatic uptake clearance was much greater than that by any other tissue. However, the physicochemical properties of Gal-PLL used for the complexation markedly affected the pharmacokinetics of the plasmid DNA complex. The clearance values demonstrate that the complexes with a larger Gal-PLL (13 or 29 kDa for the molecular weight of PLL) have a larger hepatic (target) clearance than those with a small Gal-PLL (1.8 kDa), which failed to achieve efficient delivery of plasmid DNA to hepatocytes probably due to complex dissociation before reaching the target. Similar effects of molecular size have also been obtained with galactosylated PEI [73]. Although the transfection potential was highest with PEI with the smallest molecular weight of 1.8 kDa, the polyplex composed of Gal-PEI$_{1800}$ and plasmid DNA was the least effective as far as in vivo transgene expression was concerned. Transgene expression after intravenous injection corresponded to these pharmacokinetic profiles of Gal-PLL/plasmid DNA complexes [69]. These plasmid DNA complexes with cationic vectors 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 controlling the in vivo pharmacokinetics by using a carrier molecule like Gal-PLL, the control of intracellular sorting of plasmid DNA is a good approach to increasing gene transfer at the target. Wagner et al. [74] demonstrated increased transgene expression in cultured cells following the addition of fusogenic peptides, derived from influenza virus hemmaglutinin subunit HA-2, to plasmid DNA complexes. We attached a fusogenic peptide to hepatocyte-targetable polymer and obtained improved transgene expression in the liver, indicating that the peptide also works in whole animals to, at least partially, avoid intracellular degradation [75].

Thus, the targeted delivery of polyplex can be achieved by controlling the physicochemical and biological properties of the complex, although it is sometimes hampered by its interaction with various compounds in body fluids, such as serum proteins [23]. Ogris et al. [76] have shown that when incubated with plasma, the transferrin-PEI/plasmid DNA complex undergoes aggregation, which leads to reduced delivery to the target. PEGylation appears to be a useful method for prolonging the blood circulation of a polyplex after systemic administration, and PEGylated polyplex has resulted in gene transfer to a tumor without significant toxicity after intravenous injection into tumor-bearing mice [77]. In addition to PEG, other hydrophilic polymers have been shown to extend the circulation time of polyplex [78,79].

4. Conclusion

Developing vectors that enable us to achieve transgene expression in vivo is the key issue for

successful in vivo gene therapy. Understanding the tissue distribution of plasmid DNA and its complexes is a prerequisite for designing an effective approach to improve their efficacy in vivo. Pharmacokinetic analysis will give us information about the events occurring in the body following administration and, therefore, it is a very powerful tool for developing a strategy to improve the inefficient results obtained in gene therapy trials to date.

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The hydrodynamics-based procedure for controlling the pharmacokinetics of gene medicines at whole body, organ and cellular levels

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Abstract

Hydrodynamics-based gene delivery, involving a large-volume and high-speed intravenous injection of naked plasmid DNA (pDNA), gives a significantly high level of transgene expression in vivo. This has attracted a lot of attention and has been used very frequently as an efficient, simple and convenient transfection method for laboratory animals. Until recently, however, little information has been published on the pharmacokinetics of the injected DNA molecules and of the detailed mechanisms underlying the efficient gene transfer. We and other groups have very recently demonstrated that the mechanism for the hydrodynamics-based gene transfer would involve, in part, the direct cytosolic delivery of pDNA through the cell membrane due to transiently enhanced permeability. Along with the findings in our series of studies, this article reviews the cumulative reports and other intriguing information on the controlled pharmacokinetics of naked pDNA in the hydrodynamics-based gene delivery. In addition, we describe various applications reported so far, as well as the current attempts and proposals to develop novel gene medicines for future gene therapy using the concept of the hydrodynamics-based procedure. Furthermore, the issues associated with the clinical feasibility of its seemingly invasive nature, which is probably the most common concern about this hydrodynamics-based procedure, are discussed along with its future prospects and challenges.
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Keywords: Naked plasmid DNA; Hydrodynamics-based procedure; Intravenous injection; Pharmacokinetics; Nonviral gene delivery; Gene therapy

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1. Introduction

Development of efficient gene delivery systems is undoubtedly indispensable for successful in vivo gene therapy and their unexpectedly delayed progress might be one of the main causes hindering the application of existing promising gene therapy strategies in clinical situations. The vast majority of approved vectors for current clinical protocols [1] are viral vectors thanks to their high transfection efficiency. However, they have been shown to possess immunogenic properties and the potential ability to produce mutational infection which could limit their dose and frequency of treatment. Indeed, the use of viral vectors could be discouraged by reports of the treatment-related death of a patient who had received an adenovirus vector [2] and of leukemia caused by insertional mutagenesis in patients who had undergone ex vivo transduction with a retrovirus vector [3]. In contrast, plasmid DNA (pDNA)-based nonviral vectors offer the advantages of safety and versatility and, among them, naked pDNA is expected to become one of the simplest nonviral gene medicines. It had been generally considered that naked pDNA could not produce transgene products in vivo due to its inability to enter cells and to its susceptibility to enzymatic degradation by various extracellular or intracellular nucleases [4–7]. This was the situation until Wolff et al. showed for the first time that a direct injection of naked pDNA into mouse skeletal muscle resulted in significant transgene expression with no requirement of a special delivery system [8]. Following this revolutionary report, direct needle injection of naked pDNA was shown to be applicable to various organs and tissues, such as the heart [9], liver [10], brain [11], skin [12], urological organs [13], thyroid [14] and tumors [15,16], resulting in marked transgene expression. Nonetheless, the direct injection achieves transgene expression within a limited area of cells which are close to the injection site. On the other hand, systemic injection via a vascular route would be more favorable for widespread gene delivery in that pDNA would have a greater chance of reaching many more target cells through capillary vessels. However, when applied systemically through the vascular system, naked pDNA by itself is pharmacologically inactive and produces very little transgene expression, if any, since it is rapidly scavenged and degraded by the liver nonparenchymal cells, predominantly by the liver sinusoidal endothelial cells, as demonstrated in our series of studies [17–19]. A lot of studies have attempted to overcome this unfavorable pharmacokinetic property of naked pDNA by employing various carriers such as cationic lipids or polymers [20–25]. While these approaches have resulted in some progress in terms of transgene expression efficacy, current pDNA-carrier systems are still likely to require further improvements in their in vivo pharmacokinetic aspects to overcome a lot of existing delivery barriers before they can be applied successfully to clinical gene therapy.

On the other hand, a gene delivery strategy including an intravascular injection of "large-volume" naked pDNA-containing solution has been attracting much attention lately as a novel method of controlling the in vivo pharmacokinetics of naked pDNA. Historically, successful intravascular gene delivery with carrier-free naked pDNA was pioneered by Wolff and his group. They reported that a high level of transgene expression could be obtained in mouse liver by injecting naked pDNA in hyperosmotic solution into the portal vein with transient occlusion of the outflow [26]. More recently in 1999, Liu et al. and Zhang et al. have reported an innovative finding that an astonishingly high level of transgene expression can be easily obtained in the liver and other major organs by a simple intravenous injection of naked pDNA via a tail vein with a large volume of saline at a high velocity [27,28]. This large-volume and high-velocity injection (in a typical experiment involving 20-g mice, a volume of 1.6–2.0 ml saline solution is injected over a period of 3–5 s) is a so-called hydrodynamics-based procedure [27]. Immediately after the first reports of this procedure were published, the efficiency of this technique was recognized and it was used very frequently by many researchers in a variety of fields as a simple and convenient in vivo transfection method for laboratory animals (reviewed in Liu and Knapp [29], Hagstrom [30] and Hodges and Schule [31]). The hydrodynamics-based naked pDNA delivery is very attractive compared with conventional nonviral pDNA-carrier systems in terms of efficiency, simplicity and productivity since, as shown in Fig. 1, a selective and a significant level of transgene expression can be achieved by a shot of injection of naked pDNA solution without any additional compounds being required. Furthermore, this method of gene transfer allows naked pDNA to be sufficiently effective to obtain therapeutic levels of target transgene products in vivo [32–38]. Therefore, the hydrodynamics-based procedure is strongly expected to become the key gene delivery methodology for in vivo gene therapy, although the development of non- or less-invasive practical techniques is required for its clinical application. In this article, the latest findings regarding the hydrodynamics-based procedure are reviewed including the future prospects for the clinical use of this procedure.

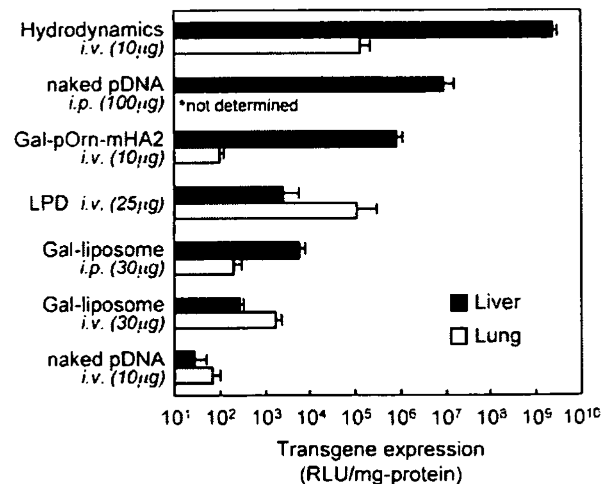

Fig. 1. Comparison of transgene expression produced by various nonviral gene delivery approaches. Mice received either hydrodynamics-based tail vein injection of naked pDNA (10 μg) [32], intraportal (i.p.) injection of naked pDNA (100 μg) in hypertonic solutions with transient occlusion of the hepatic veins [26], intravenous (i.v.) injection of pDNA (10 μg) complexed with galactosylated cationic polymer poly(L-ornithine) modified with a fusigenic peptide (Gal-pOrn-mHA2) [125], i.v. injection of pDNA (25 μg) complexed with protamine sulfate cationic polypeptide and DOTAP cationic liposomes (LPD complexes) [187], i.v. or i.p. injection of pDNA (30 μg) complexed with galactosylated liposomes (DOTMA/Chol/Gal-C4-Chol(1:0.5:0.5)) [188], or i.v. injection of naked pDNA (10 μg) in a normal volume. Luciferase activities in the liver and lung are represented as RLU per milligram extracted tissue protein. Note that the level of transgene expression depends on the formulation of the delivery carriers, the DNA constructs and doses used, and the protocol for luciferase assay, as well as the method of data processing.

2. Pharmacokinetics of DNA following intravenous injection by the hydrodynamics-based procedure

2.1. Pharmacokinetic characteristics of the hydrodynamically injected pDNA at whole body and organ levels

Immediately after the first report of the hydrodynamics-based procedure, we investigated the in vivo pharmacokinetic characteristics of naked pDNA in mice following intravenous injection of a large-volume pDNA-containing solution [18], in comparison with that of the normal procedure, i.e. an

intravenous injection of pDNA solution in a conventional "common-sense" volume (up to 200 μl for a 20-g mouse). Over the last few decades, details of the in vivo pharmacokinetics following the normal intravenous injection of naked pDNA and various DNA-related molecules have been reported by our group and others [17–19,39–44]. The time-course profile and the degree of hepatic accumulation of ^{32}P-radiolabeled pDNA were very similar for the hydrodynamics-based and normal procedures: in both procedures, pDNA was rapidly eliminated from the circulation and taken up mainly by the liver. On the other hand, the apparent plasma concentration profiles for the two procedures were significantly different: the plasma concentration of radioactivity increased with time until 10 min after the hydrodynamics-based injection. In addition, approximately 20% and 10% of the dosed radioactivity remained in the plasma pool at 30 min and 60 min, respectively, in the hydrodynamics-based procedure, while almost the entire radioactivity was eliminated from the circulation at the same time point in the normal procedure. A standard pharmacokinetic analysis of the data of pDNA injected by the hydrodynamics-based procedure could not be carried out due to possible influence in organ flow rates and the unusual plasma concentration–time profile.

The hydrodynamics-based procedure produces a marked transgene expression in various major organs including the kidney, lung, heart and spleen, and, among these, the highest expression is in the liver. The fact that luciferase activity detected in the liver is 1000- to 10 000-fold higher than that in other organs, even on a per tissue-weight basis [27,32], shows that almost all the total transgene product would be expressed in the liver since this is the biggest organ in the body. Liu et al. [27,45] speculated that the reason for this high level of transgene expression in the liver is as follows. The dynamic flow due to a high injection rate and a large volume, which is almost equivalent to the total blood volume of the animal, is likely to make the injected DNA solution accumulate in the inferior vena cava when the injection rate exceeds the cardiac output. As a result, the high hydrostatic pressure developed in the inferior vena cava will force the flow of DNA solution into tissues such as the liver, kidney and heart that are directly linked to the inferior vena cava. Since the liver is the largest organ in the body with an expandable structure, a large portion of the pDNA solution will be forced into the liver in a retrograde direction, resulting in direct exposure to the hydrostatic pressure required for a high level of transgene expression.

While the hepatic accumulation profiles of ^{32}P-radiolabeled pDNA were almost identical for the two injection procedures, marked transgene expression could be achieved by the hydrodynamics-based procedure but not by the normal one. The results of our confocal microscopic studies of liver sections indicated a widespread intrahepatic distribution of fluorescein-labeled pDNA following the hydrodynamics-based procedure. In addition, various polyanions, such as poly inosinic acid, dextran sulfate or heparin, which proved to be potent competitors for the hepatic uptake of pDNA injected by the normal procedure, did not affect the hepatic uptake of pDNA given by the hydrodynamics-based procedure [18]. These results suggest that the hepatic uptake process of the pDNA molecules is different from that following the normal intravenous injection where receptor-like mechanisms are most likely involved [17,18,44,46]. Under normal conditions, the pDNA molecules injected slowly via a tail vein with a conventional volume of solution enter the blood stream and are carried to the heart, and then distributed systemically. Due to the large amount of nucleases in the blood and in other compartments such as on the surface of tissues [46], pDNA injected by the normal procedure is likely to be rapidly degraded in the circulation and subsequently in liver cells after being recognized and taken up by liver nonparenchymal cells, especially by the liver sinusoidal endothelial cells as demonstrated recently [19]. On the other hand, part of the pDNA injected by the hydrodynamics-based procedure would be directly exposed to the liver cells and some of it would be taken up by the cells as intact molecules before being mixed with blood. This may account for the important fact that a high level of transgene expression can be obtained by the hydrodynamics-based procedure, but not by the normal one. In fact, the persistent presence of a significant level of intact pDNA was demonstrated in the liver [27]. Also, the delayed elimination of pDNA from the plasma pool may support the reduced pDNA degradation by nucleases [18].

2.2. The pDNA cellular uptake mechanisms in the hydrodynamics-based procedure

Until recently, in spite of the increasingly frequent use of the hydrodynamics-based procedure as a convenient method for functional studies of therapeutic genes or DNA elements, little is known about the mechanisms underlying efficient gene transfer by this procedure. Liu et al. [27] demonstrated in their pioneering report that a rapid injection and a large volume of pDNA solution were required to obtain a high level of transgene expression, indicating that a high blood pressure was the most critical factor for the gene transfer efficiency, and proposed that pDNA might be transported inside the liver cells by a "hydrodynamic" process. Following the first reports of the large-volume tail vein injection [27,28], Budker et al. [47] hypothesized that the cellular uptake mechanism of naked pDNA involved an active, receptor-mediated process. Their hypothesis was prompted mainly by the observations that pDNA administered by the hydrodynamics-based procedure was present around hepatocytes immediately after injection but had entered hepatocytes at 1 h after injection and that co-injection with excess polyanions, including non-expressing pDNA, sonicated salmon sperm DNA, polyglutamic acid, polycytidylic acid, polyinosinic acid, and high density lipoprotein, inhibited the transgene expression of pDNA injected under high pressure conditions. In addition, Lecocq et al. [48] demonstrated in a subcellular distribution study using differential centrifugation methods that ^{35}S-labeled pDNA remained bound to the outside surface of the plasma membrane for at least 1 h after the hydrodynamics-based procedure, supporting the hypothesis that pDNA was internalized slowly via a specific mechanism.

On the other hand, in agreement with a notion of a "hydrodynamics-based" process proposed by Liu and colleagues [27], we demonstrated in our in vivo pharmacokinetic studies of naked pDNA involving the normal or the hydrodynamics-based procedure that the hepatic uptake process appeared nonspecific [18]. Very recently, Liu et al. [49] reported that β-galactosidase and Evans Blue were efficiently delivered to the hepatocytes by the hydrodynamics-based procedure. Notably, in their electron microscopic observations, identifiable membrane defects or pores were detected in the hepatocytes, which were generated by a high-pressure solution. Based on these findings, they concluded that the hydrodynamics-based gene transfer was a nonspecific physical process and proposed the term "hydroporation" for it. At almost the same time, we also demonstrated transient hyperpermeability in the hepatic cellular membrane when using the hydrodynamics-based procedure [50]. Propidium iodide was effectively incorporated by the liver cells following the hydrodynamics-based procedure and the green fluorescent protein expressed beforehand and accumulating internally in the cytosol was dramatically eliminated from the hepatocytes following a large-volume injection of saline. These results suggested a facilitated permeation of propidium iodide and green fluorescent protein through the cell membrane, since these molecules are not supposed to cross the plasma membrane of viable cells. Nonspecificity in the cellular uptake process of pDNA was further confirmed by a competitive study. A saturable amount of empty vector did not inhibit transgene expression in the liver following the hydrodynamics-based procedure [50]. In addition, a dose-response study suggested that the transgene expression was negatively affected if the amount of injected pCMV-Luc exceeded 50 μg/mouse. This could, in part, account for the apparent inhibition of transgene expression demonstrated in Budker et al. by their report [47], where co-injection of an excess of non-expressing DNA (500 μg) led to a reduction in reporter gene (pCI-Luc; 50 μg) expression.

We have evaluated some further aspects of the enhanced membrane permeability produced by the hydrodynamics-based procedure [50]. We sought answers to the following questions: How long does the facilitated membrane permeability last? How soon does efficient intracellular delivery take place? Interestingly, a prior large-volume injection allowed significant transgene expression by a subsequent naked pDNA injection in a small volume. Sequential injections at various time intervals revealed that the effect of the hydrodynamics-based procedure appeared to be maintained for more than 15 min and the transient increase in membrane permeability recovered within 30 min. A significant level of transgene expression was observed in hepatocyte cultures isolated immediately after gene delivery,