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.

References

[1] Y. Takakura, M. Hashida, Macromolecular carrier systems for targeted drug delivery: pharmacokinetic considerations on biodistribution, Pharm. Res. 13 (1996) 820–831.

[2] M. Nishikawa, Y. Takakura, M. Hashida, Pharmacokinetic evaluation of polymeric carriers, Adv. Drug Deliv. Rev. 21 (1996) 135–155.

[3] P.J. Robinson, S.I. Rapoport, Size selectivity of blood–brain barrier permeability at various times after osmotic opening, Am. J. Physiol. 253 (1987) R459–R466.

[4] C.A. Hunt, R.D. MacGregor, R.A. Siegel, Engineering targeted in vivo drug delivery: I. The physiological and physicochemical principles governing opportunities and limitations, Pharm. Res. 3 (1986) 333–344.

[5] J.A. Wolff, R.W. Malone, P. Williams, W. Chong, G. Acsadi, A. Jani, P.L. Felgner, Direct gene transfer into mouse muscle in vivo, Science 247 (1990) 1465–1468.

[6] F. Liu, Y. Song, D. Liu, Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA, Gene Ther. 6 (1999) 1258–1266.

[7] K. Kawabata, Y. Takakura, M. Hashida, The fate of plasmid DNA after intravenous injection in mice: involvement of scavenger receptors in its hepatic uptake, Pharm. Res. 12 (1995) 825–830.

[8] R.I. Mahato, K. Kawabata, Y. Takakura, M. Hashida, In vivo disposition characteristics of plasmid DNA complexed with cationic liposomes, J. Drug Target. 3 (1995) 149–157.

[9] N. Kobayashi, M. Nishikawa, Y. Takakura, The hydrodynamics-based procedure as a unique method for controlling the pharmacokinetics of gene medicines at whole body, organ and cellular levels, Adv. Drug Deliv. Rev. 57, 713–731.

[10] M. Nishikawa, Y. Ohtsubo, J. Ohno, T. Fujita, Y. Koyama, F. Yamashita, M. Hashida, H. Sezaki, Pharmacokinetics of receptor-mediated hepatic uptake of glycosylated albumin in mice, Int. J. Pharm. 85 (1992) 75–85.

[11] M. Nishikawa, C. Miyazaki, F. Yamashita, Y. Takakura, M. Hashida, Galactosylated proteins are recognized by the liver according to the surface density of galactose moieties, Am. J. Physiol. 268 (1995) G849–G856.

[12] M. Nishikawa, F. Staud, S. Takemura, Y. Takakura, M. Hashida, Pharmacokinetic evaluation of biodistribution data obtained with radiolabeled proteins in mice, Biol. Pharm. Bull. 22 (1999) 214–218.

[13] M.J. Krantz, N.A. Holtzman, C.P. Stowell, Y.C. Lee, Attachment of thioglycosides to proteins: enhancement of liver membrane binding, Biochemistry 15 (1976) 3963–3968.

[14] D.T. Connolly, R.R. Townsend, K. Kawaguchi, M.K. Hobish, W.R. Bell, Y.C. Lee, Binding and endocytosis of glycoproteins and neoglycoproteins by isolated rabbit hepatocytes, Biochem. J. 214 (1983) 421–431.

[15] J.R. Duncan, M.J. Welch, Intracellular metabolism of indium-111-DTPA-labeled receptor targeted proteins, J. Nucl. Med. 34 (1993) 1728–1738.

[16] S.A. Ali, J.F. Eary, S.D. Warren, C.C. Badger, K.A. Krohn, Synthesis and radioiodination of tyramine cellobiose for labeling monoclonal antibodies, Nucl. Med. Biol. 15 (1988) 557–561.

[17] S. Demignot, M.V. Pimm, S.R. Thorpe, R.W. Baldwin, Differences between the catabolism and tumour distribution of intact monoclonal antibody (791T/36) and its Fab/c fragment in mice with tumour xenografts revealed by the use of a residualizing radiolabel (dilactitol-125I-tyramine) and autoradiography, Cancer Immunol. Immunother. 33 (1991) 359–366.

[18] S.R. Thorpe, J.W. Baynes, Z.C. Chroneos, The design and application of residualizing labels for studies of protein catabolism, FASEB J. 7 (1993) 399–405.

[19] P.L. Carney, P.E. Rogers, D.K. Johnson, Dual isotope study of iodine-125 and indium-111-labeled antibody in athymic mice, J. Nucl. Med. 30 (1989) 374–384.

[20] S.V. Deshpande, R. Subramanian, M.J. McCall, S.J. DeNardo, G.L. DeNardo, C.F. Meares, Metabolism of indium chelates attached to monoclonal antibody: minimal transchelation of indium from benzyl-EDTA chelate in vivo, J. Nucl. Med. 31 (1990) 218–224.

[21] O.A. Gansow, Newer approaches to the radiolabeling of monoclonal antibodies by use of metal chelates, Int. J. Radiat. Appl. Instrum., B Nucl. Med. Biol. 18 (1991) 369–381.

[22] M. Nishikawa, T. Nakano, T. Okabe, N. Hamaguchi, Y. Yamasaki, Y. Takakura, F. Yamashita, M. Hashida, Residualizing indium-111-radiolabel for plasmid DNA and its application to tissue distribution study, Bioconjug. Chem. 14 (2003) 955–961.

[23] P. Opanasopit, M. Nishikawa, M. Hashida, Factors affecting drug and gene delivery: effects of interaction with blood components, Crit. Rev. Ther. Drug Carr. Syst. 19 (2002) 191–233.

[24] V. Budker, G. Zhang, I. Danko, P. Williams, J. Wolff, The efficient expression of intravascularly delivered DNA in rat muscle, Gene Ther. 5 (1998) 272–276.

[25] F. Liu, M. Nishikawa, P.R. Clemens, L. Huang, Transfer of full-length Dmd to the diaphragm muscle of $Dmd^{mdx/mdx}$ mice through systemic administration of plasmid DNA, Molec. Ther. 4 (2001) 45–51.

[26] K.W. Liang, M. Nishikawa, F. Liu, B. Sun, Q. Ye, L. Huang, Restoration of dystrophin expression in mdx mice by intravascular injection of naked DNA containing full-length dystrophin cDNA, Gene Ther. 11 (2004) 901–908.

[27] N. Simionescu, Cellular aspects of transcapillary exchange, Physiol. Rev. 63 (1983) 1536–1579.
[28] A.E. Taylor, D.N. Granger, Exchange of macromolecules across the microcirculation, in: E.M. Renkin, C.C. Michel (Eds.), Handbook of Physiology: The Cardiovascular System IV, American Physiological Society, Bethesda, 1984, pp. 467–520.
[29] B.M. Brenner, T.H. Hostetter, H.D. Humes, Glomerular permselectivity: barrier function based on discrimination of molecular size and charge, Am. J. Physiol. 234 (1978) F455–F460.
[30] K. Mihara, T. Hojo, M. Fujikawa, Y. Takakura, H. Sezaki, M. Hashida, Disposition characteristics of protein drugs in the perfused rat kidney, Pharm. Res. 10 (1993) 823–827.
[31] F. Yuan, M. Dellian, D. Fukumura, M. Leunig, D.A. Berk, V.P. Torchilin, R.K. Jain, Vascular permeability in a human tumor xenograft: molecular size dependence and cutoff size, Cancer Res. 55 (1995) 3752–3756.
[32] T. Maack, V. Johnson, S.T. Kau, J. Figueiredo, D. Sigulem, Renal filtration, transport, and metabolism of low-molecular-weight proteins: a review, Kidney Int. 16 (1979) 251–270.
[33] K. Mihara, M. Mori, T. Hojo, Y. Takakura, H. Sezaki, M. Hashida, Disposition characteristics of model macromolecules in the perfused rat kidney, Biol. Pharm. Bull. 16 (1993) 158–162.
[34] K. Sawai, T. Miyao, Y. Takakura, M. Hashida, Renal disposition characteristics of oligonucleotides modified at terminal linkages in the perfused rat kidney, Antisense Res. Dev. (1995) 279–287.
[35] K. Sawai, R.I. Mahato, Y. Oka, Y. Takakura, M. Hashida, Disposition of oligonucleotides in isolated perfused rat kidney: involvement of scavenger receptors in their renal uptake, J. Pharmacol. Exp. Ther. 279 (1996) 284–290.
[36] Y. Yamasaki, K. Sumimoto, M. Nishikawa, F. Yamashita, K. Yamaoka, M. Hashida, Y. Takakura, Pharmacokinetic analysis of in vivo disposition of succinylated proteins targeted to liver nonparenchymal cells via scavenger receptors: importance of molecular size and negative charge density for in vivo recognition by receptors, J. Pharmacol. Exp. Ther. 301 (2002) 467–477.
[37] S. Li, W.C. Tseng, D.B. Stolz, S.P. Wu, S.C. Watkins, L. Huang, Dynamic changes in the characteristics of cationic lipidic vectors after exposure to mouse serum: implications for intravenous lipofection, Gene Ther. (1999) 585–594.
[38] A. Chonn, S.C. Semple, P.R. Cullis, Association of blood proteins with large unilamellar liposomes in vivo. Relation to circulation lifetimes, J. Biol. Chem. 267 (1992) 18759–18765.
[39] K. Furumoto, K. Ogawara, S. Nagayama, Y. Takakura, M. Hashida, K. Higaki, T. Kimura, Important role of serum proteins associated on the surface of particles in their hepatic disposition, J. Control. Release 83 (2002) 89–96.
[40] P. Opanasopit, K. Shiraishi, M. Nishikawa, F. Yamashita, Y. Takakura, M. Hashida, In vivo recognition of mannosylated proteins by hepatic mannose receptors and mannan-binding protein, Am. J. Physiol. 280 (2001) G879–G889.
[41] F. Sakurai, T. Nishioka, F. Yamashita, Y. Takakura, M. Hashida, Effects of erythrocytes and serum proteins on lung accumulation of lipoplexes containing cholesterol or DOPE as a helper lipid in the single-pass rat lung perfusion system, Eur. J. Pharm. Biopharm. 52 (2001) 165–172.
[42] F. Sakurai, T. Nishioka, H. Saito, T. Baba, A. Okuda, O. Matsumoto, T. Taga, F. Yamashita, Y. Takakura, M. Hashida, Interaction between DNA–cationic liposome complexes and erythrocytes is an important factor in systemic gene transfer via the intravenous route in mice: the role of the neutral helper lipid, Gene Ther. 8 (2001) 677–686.
[43] H. Hirabayashi, M. Nishikawa, Y. Takakura, M. Hashida, Development and pharmacokinetics of galactosylated poly-L-glutamic acid as a biodegradable carrier for liver-specific drug delivery, Pharm. Res. 13 (1996) 880–884.
[44] B.E. Houk, R. Martin, G. Hochhaus, J.A. Hughes, Pharmacokinetics of plasmid DNA in the rat, Pharm. Res. 18 (2001) 67–74.
[45] Y.K. Oh, J.P. Kim, H. Yoon, J.M. Kim, J.S. Yang, C.K. Kim, Prolonged organ retention and safety of plasmid DNA administered in polyethylenimine complexes, Gene Ther. 8 (2001) 1587–1592.
[46] M.A. Piatyszek, A. Jarmolowski, J. Augustyniak, Iodo-Gen-mediated radioiodination of nucleic acids, Anal. Biochem. 172 (1988) 356–359.
[47] J. Terebesi, K.Y. Kwok, K.G. Rice, Iodinated plasmid DNA as a tool for studying gene delivery, Anal. Biochem. 263 (1998) 120–123.
[48] E.K. Wasan, D.L. Reimer, M.B. Bally, Plasmid DNA is protected against ultrasonic cavitation-induced damage when complexed to cationic liposomes, J. Pharm. Sci. 85 (1996) 427–433.
[49] A. Bogdanov Jr., C.H. Tung, S. Bredow, R. Weissleder, DNA binding chelates for nonviral gene delivery imaging, Gene Ther. 8 (2001) 515–522.
[50] S.G. Daniel, M.E. Westling, M.S. Moss, B.D. Kanagy, FastTag nucleic acid labeling system: a versatile method for incorporating haptens, fluorochromes and affinity ligands into DNA, RNA and oligonucleotides, Biotechniques 24 (1998) 484–489.
[51] O. Zelphati, X. Liang, P. Hobart, P.L. Felgner, Gene chemistry: functionally and conformationally intact fluorescent plasmid DNA, Hum. Gene Ther. 10 (1999) 15–24.
[52] C. Neves, G. Byk, V. Escriou, F. Bussone, D. Scherman, P. Wils, Novel method for covalent fluorescent labeling of plasmid DNA that maintains structural integrity of the plasmid, Bioconjug. Chem. 11 (2000) 51–55.
[53] P.S. Slattum, A.G. Loomis, K.J. Machnik, M.A. Watt, J.L. Duzeski, V.G. Budker, J.A. Wolff, J.E. Hagstrom, Efficient in vitro and in vivo expression of covalently modified plasmid DNA, Molec. Ther. 8 (2003) 255–263.
[54] N. Zhu, D. Liggitt, Y. Liu, R. Debs, Systemic gene expression after intravenous DNA delivery into adult mice, Science 261 (1993) 209–211.
[55] Y. Liu, L.C. Mounkes, H.D. Liggitt, C.S. Brown, I. Solodin, T.D. Heath, R.J. Debs, Factors influencing the efficiency of cationic liposome-mediated intravenous gene delivery, Nat. Biotechnol. 15 (1997) 167–173.
[56] P.L. Felgner, Y. Barenholz, J.P. Behr, S.H. Cheng, P. Cullis, L. Huang, J.A. Jessee, L. Seymour, F. Szoka, A.R. Thierry, E.

Wagner, G. Wu, Nomenclature for synthetic gene delivery systems, Hum. Gene Ther. 8 (1997) 511–512.
[57] N.S. Templeton, D.D. Lasic, P.M. Frederik, H.H. Strey, D.D. Roberts, G.N. Pavlakis, Improved DNA: liposome complexes for increased systemic delivery and gene expression, Nat. Biotechnol. 15 (1997) 647–652.
[58] J.S. Remy, A. Kichler, V. Mordvinov, F. Schuber, J.P. Behr, Targeted gene transfer into hepatoma cells with lipopolyamine-condensed DNA particles presenting galactose ligands: a stage toward artificial viruses, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 1744–1748.
[59] T. Hara, Y. Aramaki, S. Takada, K. Koike, S. Tsuchiya, Receptor-mediated transfer of pSV2CAT DNA to mouse liver cells using asialofetuin-labeled liposomes, Gene Ther. 2 (1995) 784–788.
[60] S. Kawakami, S. Fumoto, M. Nishikawa, F. Yamashita, M. Hashida, In vivo gene delivery to the liver using novel galactosylated cationic liposomes, Pharm. Res. 17 (2000) 306–313.
[61] S. Kawakami, A. Sato, M. Nishikawa, F. Yamashita, M. Hashida, Mannose receptor-mediated gene transfer into macrophages using novel mannosylated cationic liposomes, Gene Ther. 7 (2000) 292–299.
[62] R.I. Mahato, S. Takemura, K. Akamatsu, M. Nishikawa, Y. Takakura, M. Hashida, Physicochemical and disposition characteristics of antisense oligonucleotides complexed with glycosylated poly(L-lysine), Biochem. Pharmacol. 53 (1997) 887–895.
[63] E. Dauty, J.S. Remy, T. Blessing, J.P. Behr, Dimerizable cationic detergents with a low cmc condense plasmid DNA into nanometric particles and transfect cells in culture, J. Am. Chem. Soc. 123 (2001) 9227–9234.
[64] G.Y. Wu, C.H. Wu, Receptor-mediated gene delivery and expression in vivo, J. Biol. Chem. 263 (1988) 14621–14624.
[65] J.C. Perales, T. Ferkol, H. Beegen, O.D. Ratnoff, R.W. Hanson, Gene transfer in vivo: sustained expression and regulation of genes introduced into the liver by receptor-targeted uptake, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 4086–4090.
[66] R. Kircheis, S. Schuller, S. Brunner, M. Ogris, K.H. Heider, W. Zauner, E. Wagner, Polycation-based DNA complexes for tumor-targeted gene delivery in vivo, J. Gene Med. 1 (1999) 111–120.
[67] C.M. Ward, M. Pechar, D. Oupicky, K. Ulbrich, L.W. Seymour, Modification of pLL/DNA complexes with a multivalent hydrophilic polymer permits folate-mediated targeting in vitro and prolonged plasma circulation in vivo, J. Gene Med. 4 (2002) 536–547.
[68] S. Li, Y. Tan, E. Viroonchatapan, B.R. Pitt, L. Huang, Targeted gene delivery to pulmonary endothelium by anti-PECAM antibody, Am. J. Physiol. 278 (2000) L504–L511.
[69] M. Nishikawa, S. Takemura, Y. Takakura, M. Hashida, Targeted delivery of plasmid DNA to hepatocytes in vivo: optimization of the pharmacokinetics of plasmid DNA/galactosylated poly(L-lysine) complexes by controlling their physicochemical properties, J. Pharmacol. Exp. Ther. 287 (1998) 408–415.
[70] N. Kobayashi, T. Kuramoto, K. Yamaoka, M. Hashida, Y. Takakura, Hepatic uptake and gene expression mechanisms following intravenous administration of plasmid DNA by conventional and hydrodynamics-based procedures, J. Pharmacol. Exp. Ther. 297 (2001) 853–860.
[71] T. Takagi, M. Hashiguchi, R.I. Mahato, H. Tokuda, Y. Takakura, M. Hashida, Involvement of specific mechanism in plasmid DNA uptake by mouse peritoneal macrophages, Biochem. Biophys. Res. Commun. 245 (1998) 729–733.
[72] Y. Takakura, T. Takagi, M. Hashiguchi, M. Nishikawa, F. Yamashita, T. Doi, T. Imanishi, H. Suzuki, T. Kodama, M. Hashida, Characterization of plasmid DNA binding and uptake by peritoneal macrophages from class A scavenger receptor knockout mice, Pharm. Res. 16 (1999) 503–508.
[73] K. Morimoto, M. Nishikawa, S. Kawakami, T. Nakano, Y. Hattori, S. Fumoto, F. Yamashita, M. Hashida, Molecular weight-dependent gene transfection activity of unmodified and galactosylated polyethyleneimine on hepatoma cells and mouse liver, Molec. Ther. 7 (2003) 254–261.
[74] E. Wagner, C. Plank, K. Zatloukal, M. Cotten, M.L. Birnstiel, Influenza virus hemagglutinin HA-2 N-terminal fusogenic peptides augment gene transfer by transferrin–polylysine–DNA complexes: toward a synthetic virus-like gene-transfer vehicle, Proc. Natl. Acad. Sci. U. S. A. 89 (1992) 7934–7938.
[75] M. Nishikawa, M. Yamauchi, K. Morimoto, E. Ishida, Y. Takakura, M. Hashida, Hepatocyte-targeted in vivo gene expression by intravenous injection of plasmid DNA complexed with synthetic multi-functional gene delivery system, Gene Ther. 7 (2000) 548–555.
[76] M. Ogris, S. Brunner, S. Schuller, R. Kircheis, E. Wagner, PEGylated DNA/transferrin-PEI complexes: reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery, Gene Ther. 6 (1999) 595–605.
[77] R. Kircheis, T. Blessing, S. Brunner, L. Wightman, E. Wagner, Tumor targeting with surface-shielded ligand–polycation DNA complexes, J. Control. Release 72 (2001) 165–170.
[78] D. Oupicky, M. Ogris, K.A. Howard, P.R. Dash, K. Ulbrich, L.W. Seymour, Importance of lateral and steric stabilization of polyelectrolyte gene delivery vectors for extended systemic circulation, Molec. Ther. 5 (2002) 463–472.
[79] F.J. Verbaan, C. Oussoren, C.J. Snel, D.J. Crommelin, W.E. Hennink, G. Storm, Steric stabilization of poly(2-(dimethylamino)ethyl methacrylate)-based polyplexes mediates prolonged circulation and tumor targeting in mice, J. Gene Med. 6 (2004) 64–75.
[80] K. Akamatsu, M. Imai, Y. Yamasaki, M. Nishikawa, Y. Takakura, M. Hashida, Disposition characteristics of glycosylated poly(amino acids) as liver cell-specific drug carrier, J. Drug Target. 6 (1998) 229–239.

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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.

Keywords: Naked plasmid DNA; Hydrodynamics-based procedure; Intravenous injection; Pharmacokinetics; Nonviral gene delivery; Gene therapy

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Contents

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.

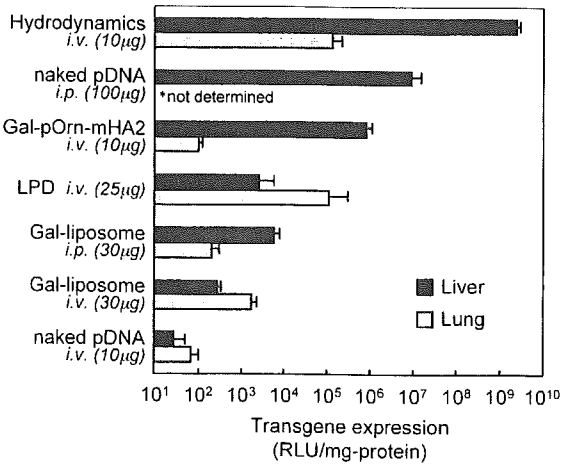

Fig. 1. Comparison of transgene expression produced by various nonviral gene delivery approaches. Mice received either hydrodynamics-based tail vein injection of naked pDNA (10 μg) [32], intraportal (i.p.) injection of naked pDNA (100 μg) in hypertonic solutions with transient occlusion of the hepatic 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,

suggesting that pDNA might be delivered intracellularly with a potential for very rapid successful transgene expression following the hydrodynamics-based procedure within the short period during which the cell membrane was affected and rendered permeable. This was supported by the findings that a hydrodynamics-based injection of luciferase-expressing RNA, which is supposed to proceed to translation once delivered intracellularly, resulted in a significant level of transgene expression as early as 10 min after injection. Moreover, Andrianaivo et al. [51] have found that the hepatocytes isolated a few minutes after a large-volume injection exhibit a marked transgene expression which was independent of the large amount of DNA remaining bound to the plasma membrane for a relatively long time. Therefore, much of the pDNA bound to the outside surface of the plasma membrane for a relatively long time [47,48] might not make a significant contribution to transgene expression. Instead, such pDNA is likely to undergo degradation by accessible external DNase or lysosomal degradation followed by internalization.

3. Application of the hydrodynamics-based procedure to therapeutic vectors and siRNA

Taking advantages of its convenience, efficiency and reproducibility, many researchers have used the hydrodynamics-based procedure as an in vivo gene transfer method in wide variety of studies which had previously been relatively difficult to perform due to the lack of efficient in vivo transfection methods. Many reports using the hydrodynamics-based procedure have already been published: the paper by Liu et al. has been cited almost 200 times in the last 5 years. As summarized in Table 1, these wide varieties of studies include evaluation of the therapeutic activities of certain genes or expressed proteins in vivo, as well as determination of the functions or efficiency of DNA regulatory elements in a novel plasmid vector. The hydrodynamics-based procedure provides us with various advantages over in vitro screening systems for specific genes or expressed proteins. A simultaneous assessment of the pharmacological, toxicological and immunological properties of certain agents can be performed in vivo, allowing us to eliminate complicated problems, such as the frequent possibility of

Table 1
Various applications of the hydrodynamics-based procedure

General purposes and genes or elements involved	References
Evaluation of biological roles and therapeutic activities of transgene products	
IL-10	[138–140]
IL-12	[35,38,141–143]
IL-15	[144]
IL-21	[144–146]
GM-CSF	[35]
Interferon (IFN)	[32]
Hepatocyte growth factor	[33,34,147–149]
Human growth hormone	[150,151]
Human flt3 ligand	[52,152]
Human flt3 and tumor necrosis factor-related apoptosis-inducing (TRAIL) ligands fusion protein	[36,153]
Human factor IX	[37,110,154]
Mannan-binding lectin	[155]
LDL receptor-GFP fusion proteins	[156]
Phenobarbital responsive unit of CYP2B	[157]
Human CYP3A4 promoter	[158]
Short-chain acyl-CoA dehydrogenase	[159]
Acticvin	[160]
Dystrophin	[133,161]
Human alpha-1 antitrypsin	[162,163]
Erythropoietin	[164]
Leptin, ciliary neurotrophic factor	[165]
Proinsulin-1	[166]
Peptide derived from fibronectin	[167]
Erythropoietin receptor-IgG_1Fc fusion protein	[168]
LDL receptor-transferrin fusion protein	[169]
Human glucocerebrosidase	[170]
Herpes simplex virus glycoprotein B (vaccination)	[171]
Evaluation and development of DNA regulatory elements	
Cell-specific promoter	[172–175]
Somatic integration	[111–113,176–179]
CpG-depleted vector	[116]
Epstein–Barr virus vectors	[105–107]
Segmental trans-splicing	[180]
DNA elements for optimized transgene expression	[97–104]
Establishment of model mouse of virus infection	
Hepatitis B virus	[54–56,181]
Hepatitis C virus	[57,182]
Hepatitis D virus	[58,183]
Delivery of non-pDNA gene medicines	
PCR-amplified product	[92,93]
Antisense oligonucleotide	[182,184]
Minicircle DNA	[117]
Bacterial artificial chromosome DNA (150-kb)	[185]
siRNA	[53,74–77,181,186]

critical differences between in vivo and in vitro, and the effects of glycosylation, and could also avoid the laborious processes involved in the synthesis and purification of the desired protein [31]. The outstandingly high level of gene transfection efficiency of the hydrodynamics-based procedure also allows in vivo expansion of particular cell populations. He et al. [52] have successfully achieved a dramatic increase in the number of functional dendritic cells and natural killer cells in vivo by a hydrodynamics-based delivery of naked pDNA encoding secreted human flt3 ligand. Furthermore, a number of challenging trials have been carried out to establish laboratory animal models of viral infection, such as hepatitis B [53–56], hepatitis C [57] and hepatitis delta [58] viruses.

3.1. Cytokine gene delivery

Among various types of application, cytokine gene delivery is one of the most promising strategies targeted at cancer gene therapy. The liver is the preferred organ as a platform for protein production to investigate the therapeutic effects of certain genes of interest in vivo, especially for secreted protein, such as cytokines, because the hepatocytes directly face the circulation over a large surface area and the hydrodynamics-based gene delivery is most efficient in the liver. We have evaluated the therapeutic activities of interferon (IFN)-γ and IFN-γ by intravenous injection of naked pDNA in mouse experimental liver and lung metastases models and demonstrated that IFN gene delivery using the hydrodynamics-based procedure serves as an effective method for in vivo or in situ cancer gene therapy [32]. So far, many studies involving cancer gene therapy have demonstrated antitumor effects of nonviral cytokine gene delivery using various carriers, such as cationic polymers or liposomes. Systemic or local administration of pDNA–cationic liposome complexes likely stimulates nonspecific induction of proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-12 and IFN-γ probably through CpG-mediated immune responses [59,60]. These cytokines are preferable as far as therapeutic efficiency is concerned due to their synergistic antitumor activities [60–63]. However, they might hamper accurate analysis of the tumor effects of the genes of interest following in vivo administration. In fact, nonspecifically produced inflammatory cytokines could account for the antimetastatic effects of lipoplexes, in part or almost completely, in some tumor models [60–63]. In one of our previous studies, we demonstrated that injection of lipoplex with IFN-noncoding pDNA (pcDNA3) resulted in the substantial production of various cytokines [64]. In contrast, very interestingly, the hydrodynamics-based procedure resulted in an efficient transgene-specific expression with little induction of nonspecific inflammatory cytokines, suggesting that nonspecific immune activation could be excluded in this procedure. This is also one of the most favorable aspects of the hydrodynamics-based procedure giving us a better way to examine the intrinsic immunological functions or antitumor activities of specific transgenes in vivo.

3.2. siRNA and siRNA-expressing vector delivery

Another interesting application of the large-volume-based approach is the delivery of small interfering RNA (siRNA) or siRNA-expressing naked vectors in vivo. RNA interference (RNAi) is nowadays well known as a powerful tool for posttranscriptional gene silencing [65–69]. In vivo application of siRNA-mediated sequence-specific cleavage of mRNA has been widely expected to be used for loss-of-function analysis of unknown or specified genes of interest as well as therapeutic purposes for the treatment of viral infections and cancers. Contrary to the time- and cost-consuming conventional strategies with knockout animals for studying gene functions in vivo, RNAi-induced suppression of endogenous gene expression is very attractive because it offers the possibility of achieving simultaneous knockdown of multiple genes or transient knockdown of lethal genes which would otherwise prevent us from investigating their functions in postnatal animals. However, in spite of increasing numbers of in vitro studies of RNAi targeting various endogenous genes, progress in the in vivo application of RNAi has been significantly delayed largely due to the lack of effective delivery systems for the pivotal role-playing siRNA. In other words, like the therapeutic gene-carrying DNAs in gene therapy, delivery issues will be the greatest challenge for RNAi-mediated therapeutics and analysis in vivo. So, successful reports demonstrating siRNA-mediated suppression of endogenous targets

are restricted to topical applications, such as direct injection into the brain or eyes and intraperitoneal delivery against grafted colon cancer cells or macrophages [70–73]. A limited number of intravascular approaches have so far produced RNAi in vivo. McCaffrey et al. and Lewis et al. were the first to observe RNAi-mediated transgene expression of exogenous luciferase genes or hepatitis B virus mRNA by the hydrodynamics-based injection of siRNA-expressing naked pDNA or synthetic siRNA [53,74,75]. Song et al. [76] and Zender et al. [77] demonstrated that frequent hydrodynamics-based injections of synthetic siRNA dramatically reduced mRNA and protein levels of the targeted gene encoding Fas receptor or caspase 8, respectively, and protected mice from liver failure and fibrosis in experimental hepatitis. Since only a few studies appear to have succeeded in producing in vivo RNAi against endogenous targets, even using the hydrodynamics-based procedure, it will be a real challenge to develop a methodology to induce RNAi effectively in vivo by systemic introduction of siRNA via the vascular route. We have also demonstrated and characterized in vivo vector-based RNAi where the hydrodynamics-based procedure was applied to a delivery method for siRNA-expressing naked pDNA targeting exogenous luciferase genes in adult mice [78]. Furthermore, we have investigated the possibility of RNAi in vivo in adult mice by the hydrodynamics-based delivery of synthetic siRNA or siRNA-expressing pDNA, targeting endogenous mdr1a/1b P-glycoprotein, an efflux pump for a wide variety of drugs, which is expressed in various somatic cells including the liver [Matsui Y, Kobayashi N, Miyagishi M, Taira K, Nishikawa M and Takakura Y, manuscript in preparation].

4. Development of gene medicine for the hydrodynamics-based procedure

Various nonviral delivery systems, such as pDNA–cationic liposome complexes (lipoplex) or pDNA–cationic polymer complexes (polyplex), have been developed [20–25] as a gene medicine for genetic or intractable diseases, but they are still to be shown to be effective in clinical situations. This is largely due to their much lower efficiency in delivering DNA molecules containing certain genes as therapeutic agents. Conventional delivery systems, even although internalized successfully by endocytosis, have to overcome the barrier posed by the endosomal trafficking process which, if not avoided, leads to degradation in lysosomes. This barrier causes a massive drop in the population of therapeutically available pDNA molecules, which face the following barriers of cytosolic metabolism [4] and nuclear membranes [79,80], markedly reducing the efficiency of conventional delivery systems.

In contrast, the hydrodynamics-based procedure allows direct cytosolic delivery of pDNA through the cell membrane as discussed above. In other words, the hydrodynamics-based procedure enables pDNA to circumvent one of the most important intracellular hurdles, i.e. passage through the cellular membrane and avoidance of endosomal or lysosomal degradation, which conventional carrier systems have to overcome for improved transgene expression [20,21,81,82]. Therefore, this represents a new opportunity that can be applied to a wide range of nucleic acid-based molecules, including naked DNA, RNA, antisense or decoy oligonucleotides [83–85], chimeric DNA–RNA oligonucleotide duplex [86–88] or peptide nucleic acid [89–91], which do not possess any natural cellular membrane-penetrating ability, to become candidate gene medicines in future gene therapies. In fact, the hydrodynamics-based procedure has been applied to the hepatic delivery of various nucleic acid-based gene medicines such as siRNA [53,74–77], PCR-amplified DNA fragments [92,93, Hirata K, Kobayashi N, Takahashi Y, Kawano H, Nishikawa M and Takakura Y, manuscript in preparation], linear DNA [94,95] and RNA [54,58], as well as naked pDNA. Furthermore, we have demonstrated the nonspecificity of hydrodynamics-based delivery by showing that macromolecules, such as bovine serum albumin and immunogloblin G, are efficiently taken up by the liver following an intravenous injection by this procedure [18]. Due to the stable, productive and easy-to-handle nature of naked pDNA, hydrodynamics-based gene delivery preferentially involves naked plasmid vectors and, hence, a lot of effort has been put into improving the DNA elements or pDNA backbone to develop the next generation of gene medicine. Although the level of

transgene expression produced by the hydrodynamics-based procedure is very high, the life of the transgene products is obviously transient [27,32,96]. Herweijer et al. [96] demonstrated that the reasons for the rapid decline in CMV promoter-driven transgene expression could be attributed to immediate promoter inactivation. Several studies have attempted to obtain long-term transgene expression by optimizing the cis-regulatory elements of DNA sequences [37,97–109]. Miao et al. [37,103,104] found that a pDNA construct containing the apolipoprotein E locus control region, liver-specific human alpha1-antitrypsin promoter, a model therapeutic human factor IX minigene sequence including a portion of the first intron, the 3′ -untranslated region and a bovine growth hormone polyadenylation signal, produced the highest serum level of human factor IX. They successfully achieved therapeutic correction of hemophilia B by the hydrodynamics-based delivery of this high-expressing human factor IX pDNA into hemophilia B mice [110]. It has also been reported that Epstein–Barr virus-based plasmid vectors containing the virus nuclear antigen 1 gene and the oriP sequence allow high and prolonged transgene expression [105–108]. Very recently, to overcome the transient nature of the transgene expression profiles, especially in dividing target cells due to loss of vector, researchers have concentrated on prolonged or semi-everlasting expression via the integration of DNA fragments to the chromosomes of the host cells. Nakai et al. [111] demonstrated that double-stranded linear DNA molecules were integrated in mouse hepatocytes in vivo in the adeno-associated virus inverted terminal repeats sequences-independent manner. In addition, it is notable that stable chromosomal integration and persistent transgene expression could be successfully achieved by Sleeping Beauty transposon–transposase vectors [112,113]. Yant et al. [113] showed that the hydrodynamics-based intravenous injection of the Sleeping Beauty transposase vector efficiently inserted transposon DNA into the mouse genome in approximately 5–6% of transfected mouse liver cells, resulting in long-term expression of human blood coagulation factor IX at a therapeutic level in a mouse model of hemophilia B. Reduction of the CpG sequences in the pDNA backbone is another intriguing approach for obtaining prolonged transgene expression. Immunostimulatory CpG motifs in pDNA are known to contribute to the acute inflammatory response, including substantial production of proinflammatory cytokines, such as IFN-γ and TNF-α, which are supposed to cause transcriptional inactivation of the CMV promoter [59,60,114]. Yew et al. [97,115,116] demonstrated that, compared with the transient expression of the unmodified vector, the novel development of CpG-depleted vectors resulted in sustained or increased expression of transgenes in mice. It has also been reported that sustained levels of transgene products could be expressed from a minicircle vector devoid of the bacterial DNA elements which play an important role in episomal transgene silencing [117,118].

The hydrodynamics-based direct cytosolic delivery also offers us the additional possibility of intravascular delivery of pDNA/functional carrier complex, which is aimed at more than just penetrating cells, as a gene medicine in place of conventional naked pDNA. Especially when focusing on other intracellular hurdles, such as cytosolic stability and nuclear transport of pDNA, we should take a second look at complexation or encapsulation of pDNA in order to overcome them. However, there are some discouraging findings showing that complexation of pDNA with some cationic molecules, such as liposomes or polymers, impedes the efficacy of transgene expression following the hydrodynamics-based procedure, indicating that the "nakedness" of the injected pDNA is an important factor [119,120, Kobayashi N, Nishikawa M and Takakura Y, unpublished observation]. However, lack of long-term transgene expression probably due to rapid promoter inactivation [96,121] would require unfavorable repeated administration. This underlines the importance of strategies, such as complexation or encapsulation of pDNA, to take advantage of these "excipients", in addition to the already mentioned "structure–activity relationship"-like improvement in DNA itself. These complexation or encapsulation strategies involve some promising carriers: e.g. polyethylenimine for increasing nuclear localization [122] or biodegradable polymer for controlled release of therapeutic pDNA [123]. One of the problems concerned with these particle-based gene delivery systems is the lack of information about the effects of the solute size on the efficacy of the hydrodynamics-based procedure which is largely influenced by the particle size although other factors such as net surface charge might also play a role. To develop

optimal strategies for a particle-based gene delivery, it is essential to clarify the particle size-dependence of the hydrodynamics-based procedure. We have recently investigated the effect of particle size on enhanced hepatic delivery via the hydrodynamics-based procedure, using polystyrene microspheres as model particles, and demonstrated that larger particles are more efficiently extravasated and trapped by the liver, whereas intracellular delivery hardly occurs if the particle size is more than putatively about 50 nm [124]. From this study, we suggested that if delivery to the extracellular spaces of hepatocyte following extravasation is enough for specific purposes, a suitable size spectrum would be in the range 50 to 500 nm or more, with larger particles being better. In view of the fact that the gyration diameter of pDNA, measured by dynamic light scattering spectrophotometery, is around 150 nm [125] and the superhelix diameter of pDNA is up to 10 nm [126], the effectively delivered population of naked pDNA leading to significant transgene expression following the hydrodynamics-based procedure is likely to cross the cellular membrane in a thread-like form or a supercoiled, relatively condensed more compact form. The interference in the transgene expression following complexation of pDNA in the hydrodynamics-based procedure is probably due to the relatively large particle size, although the effect of other factors, such as net surface charge, cannot be excluded, in that most of those complexes are not successfully delivered intracellularly but trapped in the extracellular compartment.

5. Feasibility of the clinical application of the hydrodynamics-based procedure

The hydrodynamics-based procedure seems to be a long way from application in clinical situations, since it involves a rapid intravenous injection of an extraordinarily large volume of solution; 1.6 ml of DNA-containing saline for a 20-g mouse (a typical experimental situation) corresponds to approximately 5 L of injectable solution for a 60-kg adult human. Therefore, all along, one of the major concerns about the hydrodynamics-based procedure has been its safety and invasiveness as far as its feasibility in clinical applications as a practical gene delivery technology for patients is concerned. There is no doubt that the hydrodynamics-based procedure imposes somewhat harsh conditions on the animals. However, Liu et al. [27] found that there was no indication of serious liver damage assessed by animal growth and clinical biochemistry parameters which were all within the normal ranges with the exception of a transient increase in alanine aminotransferase (ALT). Injection of a relatively large volume of pDNA solution caused no fatal damage to the mice at least in our experiments, although it was accompanied by a transient increase in serum transaminases with a rapid return to normal values within 3 days, which is consistent with the observations of other groups [27,37]. Miao et al. [37] reported that the hydrodynamics-based procedure could induce transient focal acute liver damage, involving less than 5% of the hepatocytes, which was rapidly repaired and followed by complete recovery. Herweijer et al. [96] also found that the necrotic areas affected less than 1% of the liver and only a few transgene-expressing cells died or were lost following the hydrodynamics-based procedure. Considering these findings as well as the favorable intrinsic nature of the liver with its highly capacity for regeneration, the invasive disadvantages and minor toxic effects are acceptable for therapeutic applications.

The hydrodynamics-based procedure most likely involves significant elevation of the plasma concentration of the liver enzymes, ALT and AST, even in some minimally invasive models such as the catheter-mediated approach referred to below [127], as far as substantial transgene expression is concerned. In focusing particularly on these liver enzymes and how they were affected by the hydrodynamics-based procedure, we studied the plasma concentration profiles of ALT and AST and found that the highest plasma levels of those enzymes were observed during the earlier phase of the large-volume injection [50]. This indicates that the hepatic enzymes ALT and AST immediately diffused out of the hepatocytes following the hydrodynamics-based procedure and were then eliminated gradually from the circulation depending on their pharmacokinetic characteristics. Since ALT and AST are well-known indicators of liver damage, the majority of scientists may well believe that the transient elevation of these values implies that the hydrodynamics-based procedure could be highly invasive and toxic. However, based on the facts that the increased permeability of the cell membrane is

only transient and recovers within several minutes [49,50], leakage of those enzymes is not totally due to cellular apoptosis or necrosis but is a transient event caused by the injection procedure. In other words, to obtain efficient intracellular delivery of pDNA, which is a much larger molecule than ALT and AST, it appears to be essential or a prerequisite that the hepatocyte cellular membrane is rendered permeable enough to allow transient massive effusion of the hepatic enzymes. The same could apply to the case of gene delivery via, for example, electroporation, where the electric pulses open up pores in the cell membranes through which DNA may pass into the interior [128]. Because of this, it is conceivable that cellular enzymes such as ALT and AST are not appropriate for the safety evaluation of physical gene delivery strategies, including the hydrodynamics-based procedure.

The principle of the hydrodynamics-based procedure is reproducible and applicable to an organ-restricted gene delivery method, where pDNA is injected in situ into tissue-associated vessels, as demonstrated earlier by Wolff et al. [26,129–132]. They were the first group to demonstrate the concept of organ-restricted hydrodynamics-based gene delivery (note that the "hydrodynamics-based procedure" did not exist at that time) where naked pDNA dissolved in hypertonic solutions was injected intraportally in mice whose hepatic veins were transiently occluded [26]. Zhang et al. [129] showed that substantial amounts of transgene products were obtained by an injection of hyperosmotic naked pDNA-containing solution into the afferent and efferent vessels (i.e., the portal vein, hepatic vein and bile duct) of the liver in mice, rats and dogs. They and others also successfully targeted the hindlimb muscles by giving an intraarterial large-volume injection of naked pDNA into the femoral artery in rats and even a non-human primate, the rhesus monkey [130,131,133]. In addition, the concept of hydrodynamics-based gene transfer has been applied to the rat and mouse kidney by retrograde renal vein injection of naked pDNA [134–136]. Notably, Eastman et al. [127] have very recently demonstrated the less invasive catheter-mediated hydrodynamics-based delivery of naked pDNA to individual liver lobes or the entire liver of rabbits. This is one of the examples closest to human use and the most practical technique for the future application of the hydrodynamics-based gene delivery in clinical situations. Similarly, a physiological model of hydrodynamics-based gene delivery has been used involving regional infusion of DNA-containing isotonic solutions in a precisely rate-controlled manner via branches of the rat portal vein [120]. Furthermore, hydrodynamics-based gene delivery might be clinically applicable to isolated organ grafts during organ transplantation as demonstrated recently in a rat model of liver transplantation [137].

6. Future prospects and conclusions

The hydrodynamics-based procedure is undoubtedly the most efficient of than many nonviral approaches that are currently available. This is reflected by the very frequent use of the procedure in many research studies as a convenient in vivo gene delivery method. The hydrodynamics-based procedure will surely become a common methodology for the in vivo investigation and evaluation of various gene medicines in laboratory animals. As for its clinical application, as discussed above in the last section, we believe that the hydrodynamics-based procedure, at least the concept of large-volume-mediated gene transfer, has the potential to produce a major breakthrough in the currently stagnant filed of clinical gene therapy. Undoubtedly, gene therapy is not going to become a simple daily, take-a-medicine-like treatment in the near future. Rather, the immediate future of gene therapy will probably involve those particular medications that are most likely to be used as special therapeutic options or alternatives for the treatment of intractable diseases in life-or-death situations. From this viewpoint, the marked therapeutic benefit compared with the potential risk of invasiveness, such as transient tissue damage or the accompanying surgery, should encourage the clinical application of the hydrodynamics-based procedure.

To this end, it is inevitable that the hydrodynamics-based procedure will have to incorporate some more improvements in its safety and efficiency, which can be generally classified into "hard" and "soft" types of challenges. Improvement in "hard" aspects includes development of less- or non-invasive surgical techniques and practical devices, which will enable us to reproduce hydrodynamics-based gene delivery in the human body by using an acceptable volume and rate of

intravascular injection. Use of catheter-based devices is one of the promising approaches as exemplified well by Eastman et al. [127]. Modification and optimization of gene medicines, such as pDNA, siRNA and other nucleic acid-related molecules, represent an essential improvement in "soft" aspects for the clinical stages of the hydrodynamics-based procedure. While the key requirements largely depend on the targeted diseases and the transgene products involved, the ultimate gene medicines should meet as many as possible of the following criteria: high productivity of transgene products, target cell-specificity, efficiency in terms of the number of transfected cells and the duration of transgene expression. Currently, it appears that many promising gene medicines capable of prolonged and efficient transgene expression are under development, whereas the types and number of gene-transferred cells are still passively dependent on the susceptibility of the targeted cells to the hydrodynamics-based procedure. Thus, an emphasis should be placed on developing modified gene medicines which can be controlled as far as cell-specificity and the number of transfected cells are concerned.

In conclusion, nonviral gene therapy based on the principle of the hydrodynamics-based procedure is likely to hit the headlines as a standard methodology in the near future. The development of both suitable devices and novel urgently needed gene medicines is expected soon.

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References

[1] M.L. Edelstein, M.R. Abedi, J. Wixon, R.M. Edelstein, Gene therapy clinical trials worldwide 1989–2004—an overview, J. Gene Med. 6 (2004) 597–602.

[2] T. Reid, R. Warren, D. Kirn, Intravascular adenoviral agents in cancer patients: lessons from clinical trials, Cancer Gene Ther. 9 (2002) 979–986.

[3] S. Hacein-Bey-Abina, C. von Kalle, M. Schmidt, F. Le Deist, N. Wulffraat, E. McIntyre, I. Radford, J.L. Villeval, C.C. Fraser, M. Cavazzana-Calvo, A. Fischer, A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency, N. Engl. J. Med. 348 (2003) 255–256.

[4] D. Lechardeur, K.J. Sohn, M. Haardt, P.B. Joshi, M. Monck, R.W. Graham, B. Beatty, J. Squire, H. O'Brodovich, G.L. Lukacs, Metabolic instability of plasmid DNA in the cytosol: a potential barrier to gene transfer, Gene Ther. 6 (1999) 482–497.

[5] D. Lew, S.E. Parker, T. Latimer, A.M. Abai, A. Kuwahara-Rundell, S.G. Doh, Z.Y. Yang, D. Laface, S.H. Gromkowski, G.J. Nabel, et al., Cancer gene therapy using plasmid DNA: pharmacokinetic study of DNA following injection in mice, Hum. Gene Ther. 6 (1995) 553–564.

[6] M.E. Barry, D. Pinto-Gonzalez, F.M. Orson, G.J. McKenzie, G.R. Petry, M.A. Barry, Role of endogenous endonucleases and tissue site in transfection and CpG-mediated immune activation after naked DNA injection, Hum. Gene Ther. 10 (1999) 2461–2480.

[7] H. Pollard, G. Toumaniantz, J.L. Amos, H. Avet-Loiseau, G. Guihard, J.P. Behr, D. Escande, Ca2+-sensitive cytosolic nucleases prevent efficient delivery to the nucleus of injected plasmids, J. Gene Med. 3 (2001) 153–164.

[8] J.A. Wolff, R.W. Malone, P. Williams, W. Chong, G. Acsadi, A. Jani, P.L. Felgner, Direct gene transfer into mouse muscle in vivo, Science 247 (1990) 1465–1468.

[9] H. Lin, M.S. Parmacek, G. Morle, S. Bolling, J.M. Leiden, Expression of recombinant genes in myocardium in vivo after direct injection of DNA, Circulation 82 (1990) 2217–2221.

[10] M.A. Hickman, R.W. Malone, K. Lehmann-Bruinsma, T.R. Sih, D. Knoell, F.C. Szoka, R. Walzem, D.M. Carlson, J.S. Powell, Gene expression following direct injection of DNA into liver, Hum. Gene Ther. 5 (1994) 1477–1483.

[11] T. Ono, Y. Fujino, T. Tsuchiya, M. Tsuda, Plasmid DNAs directly injected into mouse brain with lipofectin can be incorporated and expressed by brain cells, Neurosci. Lett. 117 (1990) 259–263.

[12] E. Raz, D.A. Carson, S.E. Parker, T.B. Parr, A.M. Abai, G. Aichinger, S.H. Gromkowski, M. Singh, D. Lew, M.A. Yankauckas, et al., Intradermal gene immunization: the possible role of DNA uptake in the induction of cellular immunity to viruses, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 9519–9523.

[13] J.J. Yoo, S. Soker, L.F. Lin, K. Mehegan, P.D. Guthrie, A. Atala, Direct in vivo gene transfer to urological organs, J. Urol. 162 (1999) 1115–1118.

[14] M.L. Sikes, B.W. O'Malley Jr., M.J. Finegold, F.D. Ledley, In vivo gene transfer into rabbit thyroid follicular cells by direct DNA injection, Hum. Gene Ther. 5 (1994) 837–844.

[15] T. Nomura, K. Yasuda, T. Yamada, S. Okamoto, R.I. Mahato, Y. Watanabe, Y. Takakura, M. Hashida, Gene expression and antitumor effects following direct interferon (IFN)-gamma gene transfer with naked plasmid DNA and DC-chol liposome complexes in mice, Gene Ther. 6 (1999) 121–129.

[16] G.E. Plautz, Z.Y. Yang, B.Y. Wu, X. Gao, L. Huang, G.J. Nabel, Immunotherapy of malignancy by in vivo gene transfer into tumors, Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 4645–4649.
[17] K. Kawabata, Y. Takakura, M. Hashida, The fate of plasmid DNA after intravenous injection in mice: involvement of scavenger receptors in its hepatic uptake, Pharm. Res. 12 (1995) 825–830.
[18] N. Kobayashi, T. Kuramoto, K. Yamaoka, M. Hashida, Y. Takakura, Hepatic uptake and gene expression mechanisms following intravenous administration of plasmid DNA by conventional and hydrodynamics-based procedures, J. Pharmacol. Exp. Ther. 297 (2001) 853–860.
[19] J. Hisazumi, N. Kobayashi, M. Nishikawa, Y. Takakura, Significant role of liver sinusoidal endothelial cells in hepatic uptake and degradation of naked plasmid DNA following intravenous injection, Pharm. Res. 21 (2004) 1223–1228.
[20] M. Nishikawa, L. Huang, Nonviral vectors in the new millennium: delivery barriers in gene transfer, Hum. Gene Ther. 12 (2001) 861–870.
[21] T. Niidome, L. Huang, Gene therapy progress and prospects: nonviral vectors, Gene Ther. 9 (2002) 1647–1652.
[22] N. Kobayashi, M. Nishikawa, Y. Takakura, in: B. Wang, T. Siahaan, R. Soltero (Eds.), Delivery Issues in Drug Discovery, 2005, pp. 305–319.
[23] F. Liu, L. Huang, Development of non-viral vectors for systemic gene delivery, J. Control. Release 78 (2002) 259–266.
[24] N.S. Templeton, Cationic liposome-mediated gene delivery in vivo, Biosci. Rep. 22 (2002) 283–295.
[25] M. Thomas, A.M. Klibanov, Non-viral gene therapy: polycation-mediated DNA delivery, Appl. Microbiol. Biotechnol. 62 (2003) 27–34.
[26] V. Budker, G. Zhang, S. Knechtle, J.A. Wolff, Naked DNA delivered intraportally expresses efficiently in hepatocytes, Gene Ther. 3 (1996) 593–598.
[27] F. Liu, Y. Song, D. Liu, Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA, Gene Ther. 6 (1999) 1258–1266.
[28] G. Zhang, V. Budker, J.A. Wolff, High levels of foreign gene expression in hepatocytes after tail vein injections of naked plasmid DNA, Hum. Gene Ther. 10 (1999) 1735–1737.
[29] D. Liu, J.E. Knapp, Hydrodynamics-based gene delivery, Curr. Opin. Mol. Ther. 3 (2001) 192–197.
[30] J.E. Hagstrom, Plasmid-based gene delivery to target tissues in vivo: the intravascular approach, Curr. Opin. Mol. Ther. 5 (2003) 338–344.
[31] B.L. Hodges, R.K. Scheule, Hydrodynamic delivery of DNA, Expert Opin. Biol. Ther. 3 (2003) 911–918.
[32] N. Kobayashi, T. Kuramoto, S. Chen, Y. Watanabe, Y. Takakura, Therapeutic effect of intravenous interferon gene delivery with naked plasmid DNA in murine metastasis models, Molec. Ther. 6 (2002) 737–744.
[33] J. Yang, S. Chen, L. Huang, G.K. Michalopoulos, Y. Liu, Sustained expression of naked plasmid DNA encoding hepatocyte growth factor in mice promotes liver and overall body growth, Hepatology 33 (2001) 848–859.
[34] J. Yang, C. Dai, Y. Liu, Systemic administration of naked plasmid encoding hepatocyte growth factor ameliorates chronic renal fibrosis in mice, Gene Ther. 8 (2001) 1470–1479.
[35] Z. Wang, S.J. Qiu, S.L. Ye, Z.Y. Tang, X. Xiao, Combined IL-12 and GM-CSF gene therapy for murine hepatocellular carcinoma, Cancer Gene Ther. 8 (2001) 751–758.
[36] X. Wu, Y. He, L.D. Falo Jr., K.M. Hui, L. Huang, Regression of human mammary adenocarcinoma by systemic administration of a recombinant gene encoding the hFlex-TRAIL fusion protein, Molec. Ther. 3 (2001) 368–374.
[37] C.H. Miao, A.R. Thompson, K. Loeb, X. Ye, Long-term and therapeutic-level hepatic gene expression of human factor IX after naked plasmid transfer in vivo, Molec. Ther. 3 (2001) 947–957.
[38] V.W. Lui, Y. He, L. Falo, L. Huang, Systemic administration of naked DNA encoding interleukin 12 for the treatment of human papillomavirus DNA-positive tumor, Hum. Gene Ther. 13 (2002) 177–185.
[39] W. Emlen, M. Mannik, Effect of DNA size and strandedness on the in vivo clearance and organ localization of DNA, Clin. Exp. Immunol. 56 (1984) 185–192.
[40] W. Emlen, G. Burdick, Clearance and organ localization of small DNA anti-DNA immune complexes in mice, J. Immunol. 140 (1988) 1816–1822.
[41] W. Emlen, M. Mannik, Kinetics and mechanisms for removal of circulating single-stranded DNA in mice, J. Exp. Med. 147 (1978) 684–699.
[42] T.W. Du Clos, M.A. Volzer, F.F. Hahn, R. Xiao, C. Mold, R.P. Searles, Chromatin clearance in C57Bl/10 mice: interaction with heparan sulphate proteoglycans and receptors on Kupffer cells, Clin. Exp. Immunol. 117 (1999) 403–411.
[43] V.J. Gauthier, L.N. Tyler, M. Mannik, Blood clearance kinetics and liver uptake of mononucleosomes in mice, J. Immunol. 156 (1996) 1151–1156.
[44] M. Yoshida, R.I. Mahato, K. Kawabata, Y. Takakura, M. Hashida, Disposition characteristics of plasmid DNA in the single-pass rat liver perfusion system, Pharm. Res. 13 (1996) 599–603.
[45] Y.K. Song, F. Liu, G. Zhang, D. Liu, Hydrodynamics-based transfection: simple and efficient method for introducing and expressing transgenes in animals by intravenous injection of DNA, Methods Enzymol. 346 (2002) 92–105.
[46] W. Emlen, A. Rifai, D. Magilavy, M. Mannik, Hepatic binding of DNA is mediated by a receptor on nonparenchymal cells, Am. J. Pathol. 133 (1988) 54–60.
[47] V. Budker, T. Budker, G. Zhang, V. Subbotin, A. Loomis, J.A. Wolff, Hypothesis: naked plasmid DNA is taken up by cells in vivo by a receptor-mediated process, J. Gene Med. 2 (2000) 76–88.
[48] M. Lecocq, F. Andrianaivo, M.T. Warnier, S. Wattiaux-De Coninck, R. Wattiaux, M. Jadot, Uptake by mouse liver and intracellular fate of plasmid DNA after a rapid tail vein injection of a small or a large volume, J. Gene Med. 5 (2003) 142–156.
[49] G. Zhang, X. Gao, Y.K. Song, R. Vollmer, D.B. Stolz, J.Z. Gasiorowski, D.A. Dean, D. Liu, Hydroporation as the

mechanism of hydrodynamic delivery, Gene Ther. 11 (2004) 675–682.
[50] N. Kobayashi, M. Nishikawa, K. Hirata, Y. Takakura, Hydrodynamics-based procedure involves transient hyperpermeability in the hepatic cellular membrane: implication of a nonspecific process in efficient intracellular gene delivery, J. Gene Med. 6 (2004) 584–592.
[51] F. Andrianaivo, M. Lecocq, S. Wattiaux-De Coninck, R. Wattiaux, M. Jadot, Hydrodynamics-based transfection of the liver: entrance into hepatocytes of DNA that causes expression takes place very early after injection, J. Gene Med. 6 (2004) 877–883.
[52] Y. He, A.A. Pimenov, J.V. Nayak, J. Plowey, L.D. Falo Jr., L. Huang, Intravenous injection of naked DNA encoding secreted flt3 ligand dramatically increases the number of dendritic cells and natural killer cells in vivo, Hum. Gene Ther. 11 (2000) 547–554.
[53] A.P. McCaffrey, H. Nakai, K. Pandey, Z. Huang, F.H. Salazar, H. Xu, S.F. Wieland, P.L. Marion, M.A. Kay, Inhibition of hepatitis B virus in mice by RNA interference, Nat. Biotechnol. 21 (2003) 639–644.
[54] P.L. Yang, A. Althage, J. Chung, F.V. Chisari, Hydrodynamic injection of viral DNA: a mouse model of acute hepatitis B virus infection, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 13825–13830.
[55] T. Suzuki, T. Takehara, K. Ohkawa, H. Ishida, M. Jinushi, T. Miyagi, Y. Sasaki, N. Hayashi, Intravenous injection of naked plasmid DNA encoding hepatitis B virus (HBV) produces HBV and induces humoral immune response in mice, Biochem. Biophys. Res. Commun. 300 (2003) 784–788.
[56] W.W. Chang, I.J. Su, M.D. Lai, W.T. Chang, W. Huang, H.Y. Lei, The role of inducible nitric oxide synthase in a murine acute hepatitis B virus (HBV) infection model induced by hydrodynamics-based in vivo transfection of HBV–DNA, J. Hepatol. 39 (2003) 834–842.
[57] A.P. McCaffrey, K. Ohashi, L. Meuse, S. Shen, A.M. Lancaster, P.J. Lukavsky, P. Sarnow, M.A. Kay, Determinants of hepatitis C translational initiation in vitro, in cultured cells and mice, Molec. Ther. 5 (2002) 676–684.
[58] J. Chang, L.J. Sigal, A. Lerro, J. Taylor, Replication of the human hepatitis delta virus genome is initiated in mouse hepatocytes following intravenous injection of naked DNA or RNA sequences, J. Virol. 75 (2001) 3469–3473.
[59] S. Li, S.P. Wu, M. Whitmore, E.J. Loeffert, L. Wang, S.C. Watkins, B.R. Pitt, L. Huang, Effect of immune response on gene transfer to the lung via systemic administration of cationic lipidic vectors, Am. J. Physiol. 276 (1999) L796–L804.
[60] M. Whitmore, S. Li, L. Huang, LPD lipopolyplex initiates a potent cytokine response and inhibits tumor growth, Gene Ther. 6 (1999) 1867–1875.
[61] S.W. Dow, L.G. Fradkin, D.H. Liggitt, A.P. Willson, T.D. Heath, T.A. Potter, Lipid–DNA complexes induce potent activation of innate immune responses and antitumor activity when administered intravenously, J. Immunol. 163 (1999) 1552–1561.
[62] S.W. Dow, R.E. Elmslie, L.G. Fradkin, D.H. Liggitt, T.D. Heath, A.P. Willson, T.A. Potter, Intravenous cytokine gene delivery by lipid–DNA complexes controls the growth of established lung metastases, Hum. Gene Ther. 10 (1999) 2961–2972.
[63] M. Lanuti, S. Rudginsky, S.D. Force, E.S. Lambright, W.M. Siders, M.Y. Chang, K.M. Amin, L.R. Kaiser, R.K. Scheule, S.M. Albelda, Cationic lipid:bacterial DNA complexes elicit adaptive cellular immunity in murine intraperitoneal tumor models, Cancer Res. 60 (2000) 2955–2963.
[64] F. Sakurai, T. Terada, K. Yasuda, F. Yamashita, Y. Takakura, M. Hashida, The role of tissue macrophages in the induction of proinflammatory cytokine production following intravenous injection of lipoplexes, Gene Ther. 9 (2002) 1120–1126.
[65] G. Hutvagner, P.D. Zamore, RNAi: nature abhors a double-strand, Curr. Opin. Genet. Dev. 12 (2002) 225–232.
[66] G.J. Hannon, RNA interference, Nature 418 (2002) 244–251.
[67] D.M. Dykxhoorn, C.D. Novina, P.A. Sharp, Killing the messenger: short RNAs that silence gene expression, Nat. Rev., Mol. Cell Biol. 4 (2003) 457–467.
[68] N.R. Wall, Y. Shi, Small RNA: can RNA interference be exploited for therapy? Lancet 362 (2003) 1401–1403.
[69] T. Tuschl, A. Borkhardt, Small interfering RNAs: a revolutionary tool for the analysis of gene function and gene therapy, Mol. Interv. 2 (2002) 158–167.
[70] H. Makimura, T.M. Mizuno, J.W. Mastaitis, R. Agami, C.V. Mobbs, Reducing hypothalamic AGRP by RNA interference increases metabolic rate and decreases body weight without influencing food intake, BMC Neurosci. 3 (2002) 18.
[71] S.J. Reich, J. Fosnot, A. Kuroki, W. Tang, X. Yang, A.M. Maguire, J. Bennett, M.J. Tolentino, Small interfering RNA (siRNA) targeting VEGF effectively inhibits ocular neovascularization in a mouse model, Mol. Vis. 9 (2003) 210–216.
[72] U.N. Verma, R.M. Surabhi, A. Schmaltieg, C. Becerra, R.B. Gaynor, Small interfering RNAs directed against beta-catenin inhibit the in vitro and in vivo growth of colon cancer cells, Clin. Cancer Res. 9 (2003) 1291–1300.
[73] D.R. Sorensen, M. Leirdal, M. Sioud, Gene silencing by systemic delivery of synthetic siRNAs in adult mice, J. Mol. Biol. 327 (2003) 761–766.
[74] A.P. McCaffrey, L. Meuse, T.T. Pham, D.S. Conklin, G.J. Hannon, M.A. Kay, RNA interference in adult mice, Nature 418 (2002) 38–39.
[75] D.L. Lewis, J.E. Hagstrom, A.G. Loomis, J.A. Wolff, H. Herweijer, Efficient delivery of siRNA for inhibition of gene expression in postnatal mice, Nat. Genet. 32 (2002) 107–108.
[76] E. Song, S.K. Lee, J. Wang, N. Ince, N. Ouyang, J. Min, J. Chen, P. Shankar, J. Lieberman, RNA interference targeting Fas protects mice from fulminant hepatitis, Nat. Med. 9 (2003) 347–351.
[77] L. Zender, S. Hutker, C. Liedtke, H.L. Tillmann, S. Zender, B. Mundt, M. Waltemathe, T. Gosling, P. Flemming, N.P. Malek, C. Trautwein, M.P. Manns, F. Kuhnel, S. Kubicka, Caspase 8 small interfering RNA prevents acute liver failure in mice, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 7797–7802.
[78] N. Kobayashi, Y. Matsui, A. Kawase, K. Hirata, M. Miyagishi, K. Taira, M. Nishikawa, Y. Takakura, Vector-

based in vivo RNA interference: dose- and time-dependent suppression of transgene expression, J. Pharmacol. Exp. Ther. 308 (2004) 688–693.
[79] M. Wilke, E. Fortunati, M. van den Broek, A.T. Hoogeveen, B.J. Scholte, Efficacy of a peptide-based gene delivery system depends on mitotic activity, Gene Ther. 3 (1996) 1133–1142.
[80] J. Zabner, A.J. Fasbender, T. Moninger, K.A. Poellinger, M.J. Welsh, Cellular and molecular barriers to gene transfer by a cationic lipid, J. Biol. Chem. 270 (1995) 18997–19007.
[81] C.W. Pouton, L.W. Seymour, Key issues in non-viral gene delivery, Adv. Drug Deliv. Rev. 34 (1998) 3–19.
[82] M.E. Davis, Non-viral gene delivery systems, Curr. Opin. Biotechnol. 13 (2002) 128–131.
[83] H.E. von der Leyen, R. Braun-Dullaeus, M.J. Mann, L. Zhang, J. Niebauer, V.J. Dzau, A pressure-mediated nonviral method for efficient arterial gene and oligonucleotide transfer, Hum. Gene Ther. 10 (1999) 2355–2364.
[84] N. Tomita, T. Ogihara, R. Morishita, Transcription factors as molecular targets: molecular mechanisms of decoy ODN and their design, Curr. Drug Targets 4 (2003) 603–608.
[85] Y. Shoji, H. Nakashima, Current status of delivery systems to improve target efficacy of oligonucleotides, Curr. Pharm. Des. 10 (2004) 785–796.
[86] A. Cole-Strauss, K. Yoon, Y. Xiang, B.C. Byrne, M.C. Rice, J. Gryn, W.K. Holloman, E.B. Kmiec, Correction of the mutation responsible for sickle cell anemia by an RNA–DNA oligonucleotide, Science 273 (1996) 1386–1389.
[87] L. Liang, D.P. Liu, C.C. Liang, Optimizing the delivery systems of chimeric RNA.DNA oligonucleotides, Eur. J. Biochem. 269 (2002) 5753–5758.
[88] L.W. Lai, Y.H. Lien, Therapeutic application of chimeric RNA/DNA oligonucleotide based gene therapy, Expert Opin. Biol. Ther. 1 (2001) 41–47.
[89] A. Ray, B. Norden, Peptide nucleic acid (PNA): its medical and biotechnical applications and promise for the future, FASEB J. 14 (2000) 1041–1060.
[90] P.E. Nielsen, Peptide nucleic acids: on the road to new gene therapeutic drugs, Pharmacol. Toxicol. 86 (2000) 3–7.
[91] U. Soomets, M. Hallbrink, U. Langel, Antisense properties of peptide nucleic acids, Front. Biosci. 4 (1999) D782–D786.
[92] C.R. Hofman, J.P. Dileo, Z. Li, S. Li, L. Huang, Efficient in vivo gene transfer by PCR amplified fragment with reduced inflammatory activity, Gene Ther. 8 (2001) 71–74.
[93] S. Kameda, H. Maruyama, N. Higuchi, G. Nakamura, N. Iino, Y. Nishikawa, J. Miyazaki, F. Gejyo, Hydrodynamics-based transfer of PCR-amplified DNA fragments into rat liver, Biochem. Biophys. Res. Commun. 309 (2003) 929–936.
[94] K. Rittner, A. Benavente, E. Jacobs, Ligation of exogenous linear DNA after gene transfer in vitro and in vivo, J. Gene Med. 5 (2003) 818–824.
[95] Z.Y. Chen, S.R. Yant, C.Y. He, L. Meuse, S. Shen, M.A. Kay, Linear DNAs concatemerize in vivo and result in sustained transgene expression in mouse liver, Molec. Ther. 3 (2001) 403–410.
[96] H. Herweijer, G. Zhang, V.M. Subbotin, V. Budker, P. Williams, J.A. Wolff, Time course of gene expression after plasmid DNA gene transfer to the liver, J. Gene Med. 3 (2001) 280–291.
[97] N.S. Yew, M. Przybylska, R.J. Ziegler, D. Liu, S.H. Cheng, High and sustained transgene expression in vivo from plasmid vectors containing a hybrid ubiquitin promoter, Molec. Ther. 4 (2001) 75–82.
[98] Z.L. Xu, H. Mizuguchi, A. Ishii-Watabe, E. Uchida, T. Mayumi, T. Hayakawa, Optimization of transcriptional regulatory elements for constructing plasmid vectors, Gene 272 (2001) 149–156.
[99] H. Mizuguchi, Z. Xu, A. Ishii-Watabe, E. Uchida, T. Hayakawa, IRES-dependent second gene expression is significantly lower than cap-dependent first gene expression in a bicistronic vector, Molec. Ther. 1 (2000) 376–382.
[100] M. Zabala, L. Wang, R. Hernandez-Alcoceba, W. Hillen, C. Qian, J. Prieto, M.G. Kramer, Optimization of the Tet-on system to regulate interleukin 12 expression in the liver for the treatment of hepatic tumors, Cancer Res. 64 (2004) 2799–2804.
[101] C. Notley, A. Killoran, C. Cameron, K. Wynd, C. Hough, D. Lillicrap, The canine factor VIII 3′ -untranslated region and a concatemeric hepatocyte nuclear factor 1 regulatory element enhance factor VIII transgene expression in vivo, Hum. Gene Ther. 13 (2002) 1583–1593.
[102] A. Ehrhardt, P.D. Peng, H. Xu, L. Meuse, M.A. Kay, Optimization of cis-acting elements for gene expression from nonviral vectors in vivo, Hum. Gene Ther. 14 (2003) 215–225.
[103] C.H. Miao, K. Ohashi, G.A. Patijn, L. Meuse, X. Ye, A.R. Thompson, M.A. Kay, Inclusion of the hepatic locus control region, an intron, and untranslated region increases and stabilizes hepatic factor IX gene expression in vivo but not in vitro, Molec. Ther. 1 (2000) 522–532.
[104] C.H. Miao, X. Ye, A.R. Thompson, High-level factor VIII gene expression in vivo achieved by nonviral liver-specific gene therapy vectors, Hum. Gene Ther. 14 (2003) 1297–1305.
[105] C.R. Sclimenti, A.S. Neviaser, E.J. Baba, L. Meuse, M.A. Kay, M.P. Calos, Epstein–Barr virus vectors provide prolonged robust factor IX expression in mice, Biotechnol. Prog. 19 (2003) 144–151.
[106] F.D. Cui, T. Kishida, S. Ohashi, H. Asada, K. Yasutomi, E. Satoh, T. Kubo, S. Fushiki, J. Imanishi, O. Mazda, Highly efficient gene transfer into murine liver achieved by intravenous administration of naked Epstein–Barr virus (EBV)-based plasmid vectors, Gene Ther. 8 (2001) 1508–1513.
[107] S.M. Stoll, C.R. Sclimenti, E.J. Baba, L. Meuse, M.A. Kay, M.P. Calos, Epstein–Barr virus/human vector provides high-level, long-term expression of alpha1-antitrypsin in mice, Molec. Ther. 4 (2001) 122–129.
[108] O. Mazda, Improvement of nonviral gene therapy by Epstein–Barr virus (EBV)-based plasmid vectors, Curr. Gene Ther. 2 (2002) 379–392.
[109] N.S. Yew, D.M. Wysokenski, K.X. Wang, R.J. Ziegler, J. Marshall, D. McNeilly, M. Cherry, W. Osburn, S.H. Cheng, Optimization of plasmid vectors for high-level expression in lung epithelial cells, Hum. Gene Ther. 8 (1997) 575–584.