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$_1$Fc 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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Review

Development of Cell-Specific Targeting Systems for Drugs and Genes

Makiya NISHIKAWA

Department of Biopharmaceutics and Drug Metabolism, Graduate School of Pharmaceutical Sciences, Kyoto University; Sakyo-ku, Kyoto 606—8501, Japan.

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Cell-specific targeting systems for drugs and genes have been developed by using glycosylated macromolecule as a vehicle that can be selectively recognized by carbohydrate receptors. Pharmacokinetic analyses of the tissue distribution of glycosylated proteins came to the conclusion that the surface density of the sugar moiety on the protein derivative largely determines the binding affinity for the receptors and plasma lectin. Many glycosylated delivery systems have been developed and their usefulness investigated in various settings. Galactosylated polymers, when properly designed, were found to be effective in delivering prostaglandin E_1 and other low-molecular-weight drugs selectively to hepatocytes. In addition, glycosylated superoxide dismutase and catalase were successfully developed with minimal loss of enzymatic activity. A simultaneous targeting of these two enzymes to liver nonparenchymal cells significantly prevented hepatic ischemia/reperfusion injury. On the other hand, galactosylated catalase, a derivative selectively delivered to hepatocytes, effectively inhibited hepatic metastasis of colon carcinoma cells in mice. Finally, hepatocyte-targeted *in vivo* gene transfer was achieved by synthesizing a multi-functional carrier molecule, which condenses plasmid DNA, delivering DNA to hepatocytes through recognition by asialoglycoprotein receptors, and releasing DNA from endosomes/lysosomes into cytoplasm.

Key words receptor-mediated endocytosis; pharmacokinetics; tissue distribution; asialoglycoprotein receptor; reactive oxygen species; gene delivery

The completion of the Human Genome Project allows drug candidates to be screened based on their interactions with target protein. Analysis of aberrant profiles of protein expression in disease states has led to the development and marketing of drugs able to interact with specific molecular targets. These compounds can be highly specific and effective, because they have a degree of specificity as far as their pharmacological activity is concerned. However, they still induce significant side effects and a number of adverse responses have been reported. Not only low-molecular-weight chemical compounds but also proteins, which could exhibit beneficial effects in certain diseases, have become candidate therapeutic agents to treat inherited and acquired diseases. However, most proteins examined thus far have had serious problems associated with their pharmacokinetic properties.

Targeted delivery of these drug candidates to the site of action is one solution for increasing their therapeutic index, irrespective of their specific pharmacological activity. Since Ringsdorf first proposed a model for a water-soluble macromolecular prodrug,[1] targeted delivery of anticancer drugs, biologically active proteins, antisense oligonucleotides and genes has been examined in great detail in an attempt to achieve an improved therapeutic output. In addition, the development of monoclonal antibodies as well as the finding of numbers of receptors on the cellular surface have made it possible for researchers to use such molecules involved in specific recognition as natural glycoproteins for the target-specific delivery of pharmacologically active compounds. Of the many combinations investigated, the sugar-lectin interaction has several features that are appropriate for the cell-specific targeting of pharmaceuticals; (i) the expression of lectins is specific to some types of cells, (ii) the affinity of ligands can be high enough for *in vivo* targeting, and (iii) the use of the recognition system produces little interference with the interactions that are important for life. Asialoglycoprotein receptors on hepatocytes and mannose receptors on several macrophages like Kupffer cells and liver sinusoidal endothelial cells recognize the corresponding sugars on the non-reducing terminal of sugar chains.[2] The use of sugar moieties for receptor-mediated drug targeting started with the pioneering work by Rogers and Kornfeld in 1971,[3] and a number of applications have been reported with antiviral drugs, antitumor agents, diagnostic agents, toxins, enzymes, antisense oligonucleotides and genes. Although sugar-containing delivery systems offer the possibility of improved delivery of the agent to the target when evaluated *in vitro*, some or most of the systems may not be effective *in vivo*. This is largely due to undesirable pharmacokinetic properties; for example, a highly potent antibody can bind to its antigen only when it reaches the site where the antigen is located. Therefore, in the development of cell-specific targeting system, the tissue distribution characteristics should be evaluated *in vivo* in order to investigate the various obstacles to targeted delivery such as limited passage through the endothelium, extensive uptake by mononuclear phagocyte system and rapid loss by glomerular filtration. Here, I will discuss how to develop cell-specific drug and gene targeting systems for liver cells, such as hepatocytes and liver nonparenchymal cells (Fig. 1), based on the pharmacokinetic analysis of the tissue distribution of macromolecular compounds. Then, I shall review the targeted delivery of various compounds to cells, aiming at the prevention of ischemia/reperfusion injury, inhibition of tumor metastasis and gene therapy.

PHARMACOKINETIC CONSIDERATIONS INVOLVING CELL-SPECIFIC TARGETING SYSTEMS USING GLYCOSYLATED PROTEINS

The rational design of carbohydrate receptor-mediated cell-specific targeting systems can be achieved through an understanding of the molecular mechanism governing the interaction between the receptors and ligands. The interaction of galactosylated ligands with asialoglycoprotein receptors

e-mail: makiya@pharm.kyoto-u.ac.jp
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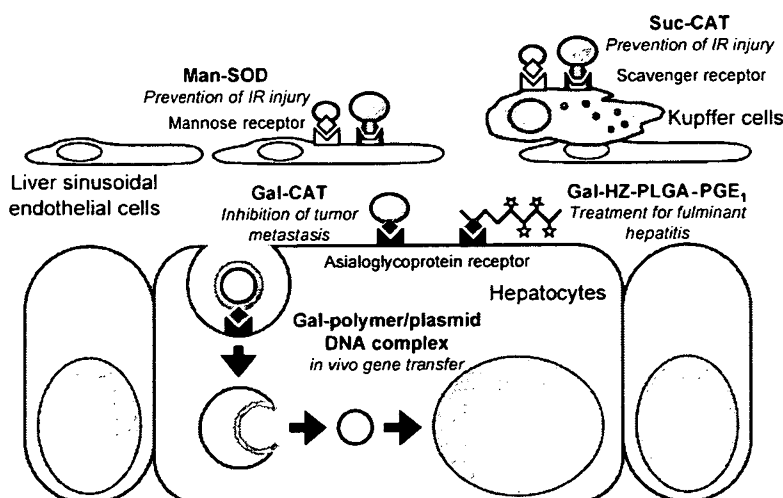

Fig. 1. Receptor-Mediated Cell-Specific Targeting of Drugs and Genes to Liver Cells

has been extensively investigated using isolated hepatocytes. Connolly *et al.* reported that highly clustered (branched) galactosides were more potent inhibitors than less clustered ones in their studies of the binding of ^{125}I-asialoorosomucoid to hepatocytes.[4] The importance of a precise geometry of the sugar chains was suggested using multi-antennary oligosaccharides.[5,6] In order to modify proteins as well as other polymers with sugar moieties, monomeric sugar derivatives are suitable because they can be easily synthesized. Experiments using mannosylated bovine serum albumin (Man-BSA)[7] and galactosylated BSA (Gal-BSA)[8] suggest that the number of sugar residues play an important role in recognition by macrophage mannose receptors or asialoglycoprotein receptors, respectively. However, the information obtained in these studies is not sufficient to design efficient targeting systems.

In addition to the affinity for the corresponding receptors, which is the major factor determining ligand-receptor interactions *in vitro*, additional factors such as blood flow rate, capillary structure and interaction with blood components[9] will affect the overall interaction with receptors *in vivo*. In the theoretical design of hepatocyte-targeted delivery systems, we have developed galactosylated proteins having different molecular weights and different numbers of galactose units, and investigated their tissue distribution in mice and rats.[10—13] During the modification procedures, attention was paid not to alter the electric charge of the proteins, because reduction in the charge sometimes increases the affinity of the protein for scavenger receptors.[14] In addition, a residualizing radiolabel using ^{111}In was used for tracing glycosylated proteins after administration, and this resulted in only minor efflux of radioactivity from tissues taking up the labeled compounds.[15—17]

After intravenous injection into mice, ^{111}In-galactosylated proteins rapidly disappeared from the plasma. They were recovered in the liver in amounts that were highly dependent on the degree of galactose modification and the administered dose. Then, the time-courses of the plasma concentration and liver accumulation of the ^{111}In-labeled galactosylated proteins were analyzed based on a physiological pharmacokinetic model, in which the body is represented by three compartments: plasma pool, the sinusoidal and Disse spaces in the liver, and the intracellular space in the liver.[10,12] The uptake of galactosylated protein in the liver was expressed as a saturable process with Michaelis–Menten kinetics having a maximum rate of uptake, $V_{max,l}$ (nmol/h), and a Michaelis constant, $K_{m,l}$ (nM). To estimate the pharmacokinetic parameters, differential equations derived from the model were simultaneously fitted to the experimental data of the plasma concentrations and liver accumulation of ^{111}In-labeled galactosylated proteins using the non-linear least-squares method MULTI associated with the Runge–Kutta–Gill method. The Michaelis constant for the hepatic uptake of ^{111}In-galactosylated recombinant human superoxide dismutase (Gal-SOD) was observed to be inversely correlated with the number of galactose residues, without a significant change in the maximum rate of uptake, $V_{max,l}$. This relationship could be successfully applied to other galactosylated proteins by using the surface density of the galactose residues as the degree of galactosylation (Fig. 2A), suggesting that this parameter controls the affinity of galactosylated proteins for asialoglycoprotein receptors. These analyses clearly demonstrated that an efficient delivery of proteins to hepatocytes by galactosylation can be achieved by adjusting the degree of galactosylation to a value for the distance between two vicinal galactose residues as short as 20—30 Å, which is of the same order as the naturally occurring sugar clusters arranged at the vertices of a triangle with sides of dimensions 15, 22, and 25 Å.[6]

A similar pharmacokinetic analysis was applied to the tissue distribution of mannosylated proteins.[18,19] We found that mannosylated proteins bind to serum-type mannan binding protein (MBP) in a structure-dependent manner. The binding to MBP was obvious at low concentrations of mannosylated proteins, and the disappearance from plasma was greatly retarded at doses less than 1 mg/kg when the molecular weight of the mannosylated proteins was 67000 or greater. Because ^{111}In-Man-BSA showed capacity-limited plasma protein binding, this binding with a maximum binding concentration (B_{max}; nM) and a dissociation constant (K_d; nM) was included in the physiological model for the analysis of the tissue distribution of ^{111}In-Man-BSA.[19] As shown in Figs. 2B, C, the $K_{m,l}$ values were fairly similar (34—68 nM) except for ^{111}In-Man$_{12}$-BSA (300 nM), whereas the K_d decreased dramatically

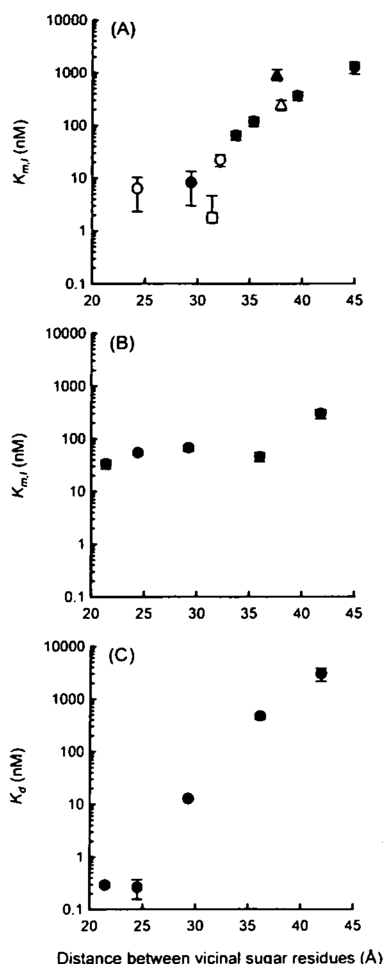

Fig. 2. Relationship between Pharmacokinetic Parameters and the Degree of Modification of ^{111}In-Glycosylated Proteins

(A) $K_{m,l}$ of ^{111}In-Gal-immunoglobulin G (□), Gal-BSA (○), Gal-SOD (●), Gal-soybean trypsin inhibitor (△), and Gal-chicken egg white lysozyme (▲). (B) $K_{m,l}$ and (C) K_d of ^{111}In-Man-BSA. Each parameter was plotted as the mean±S.D. against the average distance of two vicinal sugar residues.

on increasing the number or density of the mannose residues from 3000 nM for ^{111}In-Man$_{12}$-BSA to 0.27—0.3 nM for ^{111}In-Man$_{35}$-BSA and ^{111}In-Man$_{46}$-BSA. This suggests that the *in vivo* recognition of MBP has a stronger cluster effect than that of mannose receptors. In addition, the relationship between the density of mannose and the K_d (Fig. 2C) was comparable to that between the density of galactose on galactosylated proteins and the $K_{m,l}$ (Fig. 2A). The differences in the recognition of mannosylated ligands by hepatic mannose receptors and serum MBP could be explained by the fact that the mannose receptors contain different multiple carbohydrate recognition domains (CRDs) in the single polypeptide, whereas MBP is composed of six or more monomers with only a single CRD.[20] These findings will prove useful not only for designing cell-specific targeting systems for liver nonparenchymal cells but also for understanding the physiological roles of these lectins in the host defense system.

GLYCOSYLATED POLYMERS AS NOVEL DRUG/GENE CARRIERS

Polymers with multiple sites for conjugation with sugars as well as drugs can be more versatile carriers for drugs and genes than glycosylated proteins. Therefore, we have developed glycosylated polymers and examined their tissue distribution characteristics in mice.[21—24] In these studies, carboxymethyl-dextran, poly-L-glutamic acid (PLGA) and poly-L-lysine (PLL) were modified with galactose or mannose to obtain cell-specific targeting systems. A good correlation between the apparent hepatic uptake clearance of galactosylated PLGA (Gal-PLGA) and the number of galactose residues per PLGA was observed,[23] suggesting that the rate and extent of the delivery can be controlled by the number of galactose units as observed with galactosylated proteins. However, when the hepatic targeting of Gal-PLGA was compared with galactosylated proteins using the clearance values and the estimated surface density of galactose, it was found that Gal-PLGA requires more galactose residues to be recognized by asialoglycoprotein receptors than galactosylated proteins.[25] Cationic PLL derivatives accumulated in the liver irrespective of the modification with galactose or mannose, because of the charge-mediated interaction with both parenchymal and nonparenchymal cells in the liver.[24] However, pharmacokinetic analyses indicated that the hepatic uptake clearance of PLL increased when the polymer was modified with galactose or mannose.

Glycosylated polymers have been used for the targeted delivery of low-molecular-weight drugs and nucleotides.[25—28] Targeted delivery of vitamin K_5 by conjugating it with Gal-PLGA resulted in a rapid and continuous antihemorrhagic activity. Prostaglandin E_1 (PGE_1) was also successfully delivered to hepatocytes following its conjugation to Gal-PLGA hydrazide (Gal-HZ-PLGA) *via* a hydrazone bond. This targeting effectively inhibited the increase in plasma transaminase activity in the mouse model of fulminant hepatitis.[26]

TARGETED DELIVERY OF ANTIOXIDANT ENZYMES BY DIRECT GLYCOSYLATION: PREVENTION OF ISCHEMIA/REPERFUSION INJURY

Biologically active proteins are candidates for a variety of diseases and disorders, but most proteins have failed to exhibit any therapeutic benefits due mainly to problems associated with their pharmacokinetic properties. For instance, SOD, an antioxidant enzyme degrading superoxide anion, was regarded as a potential therapeutic agent for reactive oxygen species (ROS)-mediated diseases such as rheumatoid arthritis; however, it is rapidly cleared by glomerular filtration in the kidney, leading to a plasma elimination half-life of only 5—10 min following intravenous injection in rodents. Catalase detoxifying hydrogen peroxide, the metabolite of the superoxide anion, is also rapidly cleared by hepatocytes after intravenous injection.[29] Effective applications of these antioxidant enzymes to ROS-mediated injuries, therefore, can be achieved by their targeted delivery to sites where ROS are generated.

Ischemia/reperfusion injury is widely recognized as a significant source of morbidity and mortality in a number of clinical disorders including myocardial infarction.[30] In addi-

tion, this injury is one of the main causes of the initial poor liver function after liver transplantation. Because many of the injury-induced pathophysiological events are mediated through the production of ROS, targeted delivery of antioxidant enzymes could be useful in the treatment of various ischemia/reperfusion injuries.

The local hepatic injury associated with ischemia/reperfusion is considered to involve two phases,[31] with the initial injury being mediated by activated Kupffer cells and the subsequent injury being mediated by neutrophils that are primed during the initial period. We have tried to apply glycosylation methods as well as succinylation[32] to the delivery of SOD and catalase to the liver in a cell-specific manner, aiming at preventing hepatic ischemia/reperfusion injury.[29,33—37] Galactosylated and mannosylated derivatives of SOD and catalase have been successfully developed with enzymatic activities of 90% or higher. In addition, succinylated catalase (Suc-CAT) was also synthesized as a catalase to target liver sinusoidal endothelial cells through a scavenger receptor-mediated process. Although the ischemia following reperfusion resulted in a striking increase in serum transaminase activity, the administration of SOD and catalase derivatives significantly inhibited this. Among various combinations, Suc-CAT and Man-SOD showed the greatest inhibitory effect against the injury evaluated by serum transaminase activity and by the integrity of the liver tissues.[36] This combination also significantly suppressed the expression of intercellular adhesion molecule-1 along the hepatic sinusoids and prevented neutrophil infiltration in the liver.[37] The numbers of mannose receptors and scavenger receptors and the affinity of these derivatives for the receptors suggest that the liver sinusoidal endothelial cells have a higher level of catalase activity than SOD activity, whereas Kupffer cells have mainly SOD activity. Therefore, a plausible mechanism of the protection by Suc-CAT and Man-SOD is the dismutation of superoxide anion that Kupffer cells generate by Man-SOD, followed by Suc-CAT-mediated elimination of hydrogen peroxide, which is a stable amphiphilic molecule that can diffuse through the cellular membrane. Thus, this 'double targeting' of SOD and catalase to liver nonparenchymal cells appears to be a promising approach to reducing the ROS produced by Kupffer cells and neutrophils infiltrating tissue.

INHIBITION OF TUMOR METASTASIS BY TARGETED DELIVERY OF CATALASE

Although high levels of ROS are cytotoxic, as indicated in hepatic ischemia/reperfusion injuries, low levels of ROS act as second messengers in the activation of cellular responses. It has been reported that ROS are involved in the regulation of the expression levels of adhesion molecules and matrix metalloproteinases (MMPs).[38] Furthermore, a low level of hydrogen peroxide increases cell proliferation. Therefore, detoxification of ROS, especially hydrogen peroxide, would be beneficial for the prevention of tumor metastasis. Some studies have already shown significant, but limited, effects of antioxidant enzymes on experimental tumor metastasis in animal models,[39,40] but no attempts have been made to achieve their targeted delivery.

Therefore, we examined the effects on tumor metastasis of targeted delivery of catalase to the liver and lung, two major sites of metastatic events.[41—43] An intraportal injection of 1×10^5 mouse colon carcinoma colon 26 cells resulted in the formation of more than 50 metastatic colonies on the surface of the liver at 14 d after injection. An intravenous injection of catalase (CAT; 35000 units/kg of body weight) significantly reduced the number of colonies in the liver. Among the catalase derivatives examined, Gal-CAT showed the greatest inhibitory effect on hepatic metastasis, and the number of colonies was significantly smaller than that following treatment with catalase, Man-CAT or Suc-CAT.[42] High activity of MMPs, especially MMP-9, were detected in the liver of mice bearing metastatic tumor tissues, and this was significantly reduced by Gal-CAT. The *in situ* zymography suggested that the gelatinase activities in the tumor-bearing liver were close to the sinusoids of the liver. In addition, the gelatin zymographic analysis of liver homogenates clearly demonstrated that MMP-9 is the major contributor to gelatinolysis in tumor-bearing mouse liver after intraportal inoculation of colon 26 tumor cells. Further studies are needed to identify which cells in the liver contribute to the gelatinase activity in tumor-bearing liver, because MMPs are known to be produced from various cells including tumor cells, endothelial cells, macrophages and hepatocytes. Our preliminary results obtained using cultured hepatocytes and colon 26 tumor cells show that, under oxidative stress, MMP-9 is largely produced by hepatocytes, whereas MMP-2 is from colon 26 cells. These findings suggest that hepatocytes are the major source of the MMPs detected in the liver, which supports the experimental data showing that Gal-CAT, the hepatocyte-targeting type, has the greatest inhibitory effect on tumor metastasis in the liver.

APPLICATION OF CELL-SPECIFIC TARGETING SYSTEMS TO NONVIRAL GENE DELIVERY

The *in vivo* gene transfer profile required for effective gene therapy depends on the target disease.[44] Target cell-specific gene transfer is important for various aspects of *in vivo* gene therapy, because transgene expression in non-target cells could lead to the induction of side-effects. Generally speaking, plasmid DNA-based nonviral vectors offer the advantages of safety and versatility over viral vectors.[45] So far, several promising results of gene transfer using plasmid DNA-based approaches have been reported in preclinical and clinical settings.[46]

Gene transfer is expected to occur in cells reached by vectors directly or *via* the blood circulation. To achieve target cell-specific gene transfer, a variety of approaches has been examined, from the selection of the administration route of the vector to the use of tissue-specific promoters. Among those, targeted delivery of plasmid DNA using a receptor-mediated process would be an ideal approach. Plasmid DNA is a huge macromolecule with a strong negative charge and, therefore, its tissue distribution is highly restricted (Fig. 3).[45,47] The uptake by Kupffer cells and liver sinusoidal endothelial cells *via* a scavenger receptor-like mechanism largely determines its tissue distribution following intravascular administration of plasmid DNA in its naked form.[48,49]

Complex formation with positively charged molecules is an easy way to reduce the negative charge of plasmid DNA. We have synthesized a series of glycosylated polymers to

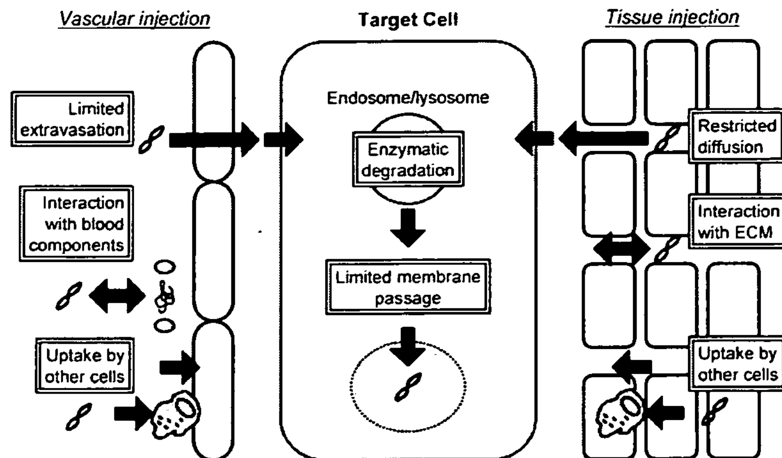

Fig. 3. Delivery Barriers for Nonviral Vector in Target Cell-Directed *in Vivo* Gene Transfer

achieve cell-specific gene transfer to carbohydrate receptor-positive cells.[50—53] Galactosylated PLL (Gal-PLL) synthesized using PLL with a molecular weight of 1800, 13000 or 29000 was mixed with plasmid DNA to form complexes.[50] A larger amount of PLL_{1800} was required for complex formation than with PLL_{13000} and PLL_{29000}, and increasing the number of galactose units on Gal-PLL resulted in reduced binding to plasmid DNA. The particle size and zeta-potential of the complexes varied depending on the mixing ratio and Gal-PLL used, and plasmid DNA/Gal-PLL complexes having diameters of 200 nm or less and a weak negative charge were prepared. After intravenous injection of ^{32}P-plasmid DNA/Gal_{13}-PLL_{13000} and ^{32}P-plasmid DNA/Gal_{26}-PLL_{29000}, almost 80% of the radioactivity rapidly accumulated in the liver, preferentially in hepatocytes. Compared with these complexes, ^{32}P-plasmid DNA/Gal_5-PLL_{1800} and ^{32}P-plasmid DNA/Gal_5-PLL_{13000} had a smaller hepatic clearance, suggesting that both the molecular weight of PLL and the degree of galactose modification determine the hepatic targeting of plasmid DNA. *In vitro* and *in vivo* gene expression studies showed that plasmid DNA/Gal_{13}-PLL_{13000} and plasmid DNA/Gal_{26}-PLL_{29000} complexes were superior to plasmid DNA/Gal_5-PLL_{1800} complex for introducing the DNA into cells. Thus, targeted delivery of plasmid DNA to hepatocytes *in vivo* was successfully carried out by controlling both the physicochemical properties of the carrier, Gal-PLL, and the particulate properties of the plasmid DNA/Gal-PLL complexes. The results obtained clearly indicate that the molecular weight and degree of galactose modification of Gal-PLL are major factors determining the stability of DNA/carrier complex formation; this in turn determines the cell-specific targeting and transgene efficiency.

However, the level of transgene expression by targeted gene delivery does not appear to be sufficient for treating diseases. To increase the expression efficiency by a nonviral approach, compounds that can enhance transgene expression, such as viruses or viral proteins, fusogenic lipids, and fusogenic, and membrane-disruptive peptides have been introduced into nonviral carrier systems. To preserve the advantages of nonviral systems, only synthetic compounds like fusogenic peptides are candidate helper molecules. In addition, from a pharmacokinetic point of view, they should be firmly attached to the delivery system. Based on these criteria, we have tried to improve the efficiency of transgene expression by synthesizing a multi-functional carrier molecule, galactosylated poly-L-ornithine (pOrn)-fusogenic peptide conjugate.[51] This molecule was designed so that it would (i) bind and condense DNA to optimize the systemic disposition profile, (ii) deliver DNA to hepatocytes through asialoglycoprotein receptor recognition, and (iii) release DNA from endosomes/lysosomes into cytoplasm after internalization. To this end, a cationic pOrn was modified first with galactose, then with a fusogenic peptide (mHA2) to obtain Gal-pOrn-mHA2. When applied with Gal-pOrn-mHA2 to HepG2 cells, an asialoglycoprotein receptor-positive cell line, fluorescein-labeled plasmid DNA showed a diffuse profile, suggesting the release of plasmid DNA from the endosome/lysosome compartment. A large amount of transgene product was obtained in the liver of mice injected with plasmid DNA/Gal-pOrn-mHA2 complex, which was much greater than that obtained with plasmid DNA/Gal-pOrn or plasmid DNA/cationic liposome complex. The luciferase activity in hepatocytes accounted for more than 95% of the total activity in all the tissues examined. Thus, hepatocyte-targeted *in vivo* gene expression was achieved by the intravenous injection of DNA complex with the multi-functional gene carrier, which can be an effective therapeutic option for hepatic diseases in which any important genes in hepatocytes are missing or mutated.[54]

CONCLUSION

Cell-specific targeting of drugs and genes is a promising approach not only for increasing the therapeutic benefits but also for reducing the side-effects. Galactosylation and mannosylation of macromolecules have been proved to be effective in delivering a variety of pharmaceuticals from low-molecular-weight drugs such as PGE_1 to huge plasmid DNA. Careful examination of the tissue distribution of such delivery systems after *in vivo* administration is essential for the theoretical development of effective delivery systems, because there are many delivery barriers especially for nonviral vectors.[45] Therefore, analytical methods for the tissue distribution of macromolecular compounds are also important for the design of cell-specific targeting systems. We recently de-

veloped a novel radiolabeling method for plasmid DNA that is suitable for the analysis of the tissue distribution of plasmid DNA and its complexes.[55] Finally, it is fully expected that further basic studies on cell-specific targeting will lead to applications in routine medical practice in the near future.

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