Systemic Administration of TerplexDNA System: Pharmacokinetics and Gene Expression

Lei Yu,[1] Hearan Suh,[1] Jae Joon Koh,[1] and Sung Wan Kim[1,2]

Received April 4, 2001; accepted May 24, 2001

Purpose. The aim of this study is to extend our previous studies to investigate the TerplexDNA synthetic gene carrier system in pharmacokinetics, biodistribution, and gene expression in major organs after systemic administration.

Methods. The stability of the TerplexDNA system was analyzed *in vitro* with a serum incubation assay. The TerplexDNA PK/PD studies were conducted by quantitation of Terplex/radiolabeled DNA [CTP α-^{32}P] complexes after rat-tail vein injection. The effect of the TerplexDNA system on gene expression in mouse major organs was analyzed by measuring luciferase activities after systemic administration.

Results. The TerplexDNA gene carrier showed significantly longer retention in the vascular space than naked plasmid DNA alone. At early time points (1 h postvenous injection), the lung was the major organ of the TerplexDNA distribution, followed by the liver as a major distribution organ at later time points (24 h postinjection). The major organs of transgene expression after intravenous injection were the liver and heart.

Conclusion. The TerplexDNA system has the potential for *in vivo* applications due to its higher bioavailability of plasmid DNA in the tissues, and due to its organ specific distribution.

KEY WORDS: gene delivery; gene therapy; gene carrier; PK/PD; TerplexDNA system.

INTRODUCTION

The emphasis of 21st-century medicine will shift from treating the sick to treating individuals predisposed to certain diseases. Gene therapy is a novel therapeutic method that is currently defined as the transfer of nucleic acid —either RNA or DNA—to human tissue or cells to treat or prevent both inherited and acquired diseases (1).

The center of gene therapy is gene transfer, or delivery of genes into the desired cells followed by gene expression. Gene vectors, or gene carriers, are necessary tools used to mediate gene transfer into human cells (2). Usually, there are two classes of gene vector systems: virus-based gene vector system and non-virus-based gene carrier system. Safety issues have severely limited the application of viral vectors to patients (3). Thus, the development of effective and safe non-viral gene carrier systems has become more urgent and important than ever before. Currently, the most commonly used non-viral vector systems are lipid-based (lipoplex), synthetic polymer-based (polyplex), and lipid-polymer-based (lipopolyplex) gene carrier systems with ligands for specific cell surface receptors and intracellular trafficking function (4).

The naked DNA technique has a very low efficiency of gene transfer *in vivo* even though it is simple and safe. Non-viral gene carrier systems can serve as enhancers for gene transfection due to their protection of plasmid DNA from degradation *in vivo* and facilitation of the uptake of plasmid DNA into cells. Most non-viral gene carriers form complex by condensing plasmid DNA via ionic interaction. These systems have several properties that are superior to the viral vector systems, such as the following: (a) no biosafety problems even though some non-viral gene carrier system showed cytotoxicity in cell studies; (b) there is little restriction on the size of the DNA to be transfected; (c) most of non-viral gene carriers have shown negligible immunogenecity and thus can be repeatedly applied without the immune system becoming sensitized; (d) the size and charge of the overall complex can be optimized by varying the amount of cationic molecules to the amount of plasmid DNA; and (e) the functional groups necessary to the gene delivery pathway or enhancing gene delivery efficiency can be easily incorporated into the gene carrier backbone by chemical conjugation or physical interaction. In most cases, the transfecting complex is formed spontaneously when mixing the purified DNA solution with the cationic lipids or polyamines.

The lipopolyplex gene carrier system combines the advantages of both lipid-based and cationic polymer-based gene carrier systems. It condenses DNA efficiently, reduces the size of DNA/polymer/lipid complexes smaller than lipid-DNA alone, and increases the complex stability *in vivo*. Recently, we developed a novel synthetic gene carrier system, the TerplexDNA, which showed high gene transfer efficiency *in vitro* and *in vivo* myocardium gene transfers (5–9). We called it TerplexDNA system because it is composed of three major components in the system: stearyl-poly L-Lysine (stearyl-PLL), low-density lipoprotein (LDL), and DNA (7,9). The TerplexDNA system is based on a balance of hydrophobic and electrostatic interactions between LDL, stearyl-PLL, and DNA. Each component in the TerplexDNA system plays an important role in the complex formation and in gene delivery. The ε-amino groups on the PLL component complexes with the negatively charged phosphates on the DNA, partially neutralizing the charge. The stearyl groups on PLL insert into the core of the lipid of LDL with hydrophobic interactions. These interactions make the TerplexDNA system stable *in vitro* and *in vivo*. The TerplexDNA is a lipopolyplex system. LDL incorporation into the polymer gene carrier system could enhance gene delivery efficiency probably due partly to the augmentation of the LDL receptor-mediated endocytosis pathway.

The information of the behavior of gene carrier in the body is extremely important for gene carrier *in vivo* applications because the pharmacokinetics and bioavailability of DNA or gene carrier can be affected by many biologic and physicochemic factors once they are systemically administrated. The pharmacokinetic profiles of a particle within the body are governed by its physicochemic properties such as its size, surface charge, and morphology as well as its interaction with serum proteins and cell surfaces.

[1] Center for Controlled Chemical Delivery, Department of Pharmaceutics and Pharmaceutical Chemistry, University of Utah, Salt Lake City, Utah 84112.

[2] To whom correspondence should be addressed. (e-mail: rburns@deans.pharm.utah.edu)

0724-8741/01/0900-1277$19.50/0

In this study, we investigated the pharmacokinetics, biodistribution, and *in vivo* gene expression in major organs after systemic administration of naked or the Terplex [^{32}P] DNA in rats and mice. The gene expression was evaluated by measurement of luciferase activities in cell lysates from different organs at various time points after intravenous injection.

MATERIALS AND METHODS

Materials

Plasmid pCMV-Luc encoding firefly luciferase driven by human cytomegalovirus (CMV) immediate early gene promoter/enhancer was constructed and described previously (7). The luciferase substrate solution was purchased from Promega (Madison, WI). LDL and poly-L-lysine (M_r 50,000) were purchased from Sigma (St. Louis, MO). All the medium and cell culture reagents were purchased from GIBCO Scientific Life (Rockville, MD).

Preparation of DNA/Polymer Complex (TerplexDNA)

The formation, characterization, and identification of the TerplexDNA system were performed as described in previous studies (5,6,9). The plasmid DNA was amplified in *Escherichia coli* DH5α cells, isolated with "Endofree" plasmid amplification and purification kit (Qiagen), and reconstituted in phosphate-buffered saline. Plasmid purity was determined by spectrophotometry at A260/A280 and by 0.8% agarose gel electrophoresis.

TerplexDNA System Nuclease Protection Assay

The protective effect of Terplex system for the DNA from nuclease degradation was studied using rat serum incubation assay. Briefly, the TerplexDNA complexes or naked plasmid DNA (1 μg) was incubated with freshly prepared rat serum at 37°C for various time points. At indicated times, an aliquot of the reaction was removed and immediately mixed with equal volume of 100 mM EDTA (pH 8) to stop the reaction. The plasmid DNA was isolated from the gene carrier by phenol-chloroform-isoamylalcohol methods and analyzed by gel electrophoresis.

Radiolabeling of Plasmid DNA

Plasmid DNA was radiolabeled with [CTP α-^{32}] (ICN, Costa Mesa, CA) using nick translation system (Promega, Madison, WI) according to the manufacturer's instruction manual. For the determination of radioactivity of labeled probe, 2 μl of the samples were added to 98 μL of STE buffer (pH 8, 150 mM NaCl, 10 mM Tris-HCl, pH 8, and 1mM EDTA) and mixed with scintillant (Hionic Fluor™, Packard, Netherlands) in scintillation vials. The radioactivity of the samples were measured with liquid scintillation counter (TRI-CARB 1900 TR, Packard, Downers Grove, IL) after they were thoroughly mixed.

Pharmacokinetic and Biodistribution Studies

Male Sprague–Dawley rats (250–300 g) were purchased from Simonsen (Gilroy, CA). One day prior to the experiment, rats underwent external jugular vein cannulation under ketamine:acepromazine:xylazine cocktail (50:3:3 mg/kg) anesthesia. Groups of six rats were injected intravenously via a cannula with 10 pmol of [^{32}P] naked plasmid DNA or the TerplexDNA. The cannula was flushed with saline immediately after injection. At indicated times, blood was taken from the jugular vein via cannula, and the rats were sacrificed at 5 min, 1 h, or 24 h by injecting barbiturate overdose. Before harvesting organs, the whole body was perfused with saline into the inferior vena cava until the eluate was clear in order to exclude the amount of DNA inside the various organ blood pool. Samples of the lung, heart, liver, spleen, and kidney were collected, rinsed with saline and blot-dried. The weighed amounts of tissues and blood were solubilized in Soluable™ (Packard, Netherlands), decolorized with 30% hydrogen peroxide, mixed with aqueous counting scintillant (Hionic-Fluor™, Packard, Netherlands) and counted for ^{32}P radioactivity on a liquid scintillation β counter (TRI-CARB 1900 TR, Packard, Downers Grove, IL).

Pharmacokinetic Data Analysis

Serum concentration-versus-time data were fitted to a two-compartment model using SCIENTIST™ pharmacokinetic data analysis program and represented by following the biexponential equation:

$$C = C_1 * \exp(-\lambda_1 t) + C_2 * \exp(-\lambda_2 t)$$

The reciprocal of the measured serum concentration values was found to be appropriate as weighing factors. The area under serum concentration-time curves ($AUC = C_1/\lambda_1 + C_2/\lambda_2$) and area under the first moment curve ($AUMC = C_1/\lambda_1^2 + C_2/\lambda_2^2$) were calculated from the slopes and coefficients. Systemic clearance (CL_T) was calculated from Dose/AUC, mean residence time (MRT) from AUMC/AUC, steady-state volume of distribution (Vd_{ss}) from $CL_T \times MRT$, and half-life ($t_{1/2}$) was calculated from $0.693/\lambda_2$;.

In Vivo Luciferase Gene Expression Assay

The TerplexDNA complex or naked plasmid DNA (pCMV-Luc) was intravenously injected into Balb/c mice (5–6 weeks old) via tail vein at a dose of 15 μg of DNA/animal in total volume of 300 μL. At indicated times, each was anesthetized as described above and the whole body of the animals were perfused with normal saline. Organs including the heart, lung, liver, spleen, and kidney were harvested. The freshly dissected organs were homogenized by using homogenizer (Biospec Products Inc., Bartlesville, Oklahoma) with 2 volumes of lysis buffer at 30,000 rpm on ice. For luciferase activity assay, the cells transfected with plasmid pCMV-Luc were lysed with lysis buffer (1% Triton X-100, 100 mM K_3PO_4, 2 mM dithiothreitol, 10% glycerol, and 2 mM EDTA pH 7.8) for 15 min at room temperature. A 20-μL aliquot of cell lysate was then mixed with 50 μL of luciferase assay reagent at room temperature and inserted in the luminometer. Light emission was measured in triplicates over 10 s and expressed as relative light units, which were normalized to the protein content of each sample, which determined by BCA protein assay (Pierce, Rockford, IL). All the experiments were conducted triplicately. Each data point was obtained using 5 mice (n = 5).

RESULTS

The Effect of the TerplexDNA System on DNA Protection

The protective effect of the TerplexDNA system on plasmid DNA from nuclease degradation was evaluated by incubation of the TerplexDNA complexes with freshly prepared rat serum. In the serum incubation assay, gel electrophoresis was used for analysis of plasmid DNA integrity. By the addition of rat serum to the TerplexDNA system solution or to the naked plasmid DNA solution, the naked DNA was completely degraded into small fragments 5 min after incubation in rat serum. On the other hand, significant amounts of plasmid DNA still remained in the TerplexDNA samples 120 min after incubation in rat serum. The dominant conformation of plasmid DNA on the gel is linear/open circular type due to vigorously vortexing the plasmid DNA in organic solvent for separation of plasmid DNA from the TerplexDNA system (Fig. 1).

TerplexDNA System Pharmacokinetic Studies

The pharmacokinetic studies of the Terplex [α-^{32}P] DNA and naked [α-^{32}P] DNA in blood were carried out following rat intravenous injection. The pharmacokinetic parameters calculated using area moment analysis demonstrate significant differences in the disposition of plasmid DNA between the two cases. The TerplexDNA gene carrier was more slowly eliminated from body (Table I). The TerplexDNA showed much higher steady-state volume of distribution (Vd_{ss}) than naked DNA. The high value of Vd_{ss} suggests that the plasmid DNA, especially the TerplexDNA, distributed into the intracellular space as well as vascular and interstitial space. The presence of intact reporter genes in tissues following intravenous injection of the TerplexDNA or naked plasmid DNA was evaluated by PCR (data not shown) and by major organ gene expression (see next).

Table I. Pharmacokinetics and Biodistribution Studies

	Clearance (L/h)	Half-life (h)	Mean residual time (h)	Steady-state volume of distribution (L/kg)
Terplex/DNA	0.116	6.18	7.84	2.61
Naked DNA	0.132	1.96	2.54	0.95

TerplexDNA System Biodistribution Studies

Distribution of plasmid DNA in mice was assessed by measuring the radioactivity in selected tissues after intravenous injection of radioisotope labeled plasmid DNA with or without the TerplexDNA gene carrier (Fig. 2). Rapid clearance of plasmid DNA from circulation was observed with extensive distribution into the organs after the TerplexDNA and naked DNA administrations. After the naked DNA administration, great portions of the recovered dose of DNA were found in the kidney and liver (>60%), although the relative distribution profiles were changed over time. In the kidney, the amount of plasmid DNA declined over time up to 24 h post-injection whereas the amount of DNA increased in the liver and spleen. Relative distribution profiles were altered with the TerplexDNA administration. Naked plasmid DNA was preferably distributed into the lung at early time point (1 h post-injection) (Fig. 2A), but the amount of DNA in the liver increased continuously and became a major disposition organ 24 h after injection (Fig. 2B). The DNA concentration in the kidney remained relatively high throughout the experimental period (Fig. 2).

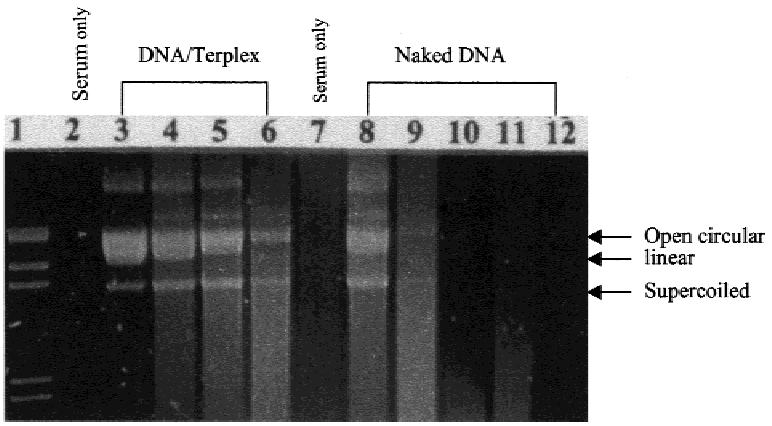

Fig. 1. Effect of the TerplexDNA system on protection of plasmid DNA from rat serum attack. Plasmid DNA (TerplexDNA or naked) was incubated in freshly prepared rat serum at 37°C for the indicated period of time. The reactions were stopped by addition of EDTA. Plasmid DNA was extracted using phenol/chloroform/isoamyl alcohol mixture methods. The integrity of extracted DNA was evaluated using electrophoresis on 1% agarose gel. Lane 1 (molecular weight marker, λ DNA/Hind III Fragment); lane 2 (serum only); lane 3 to lane 6 show the time course of incubation of the TerplexDNA system in rat serum. Lane 3 (10 min); lane 4 (30 min); lane 5 (60 min); lane 6 (120 min. Lanes 8 to 12 show the time course of incubation of naked plasmid DNA in rat serum. Lane 7 (serum only); lane 8 (2 min); lane 9 (5 min); lane 10 (10 min); lane 11 (20 min); lane 12 (30 min).

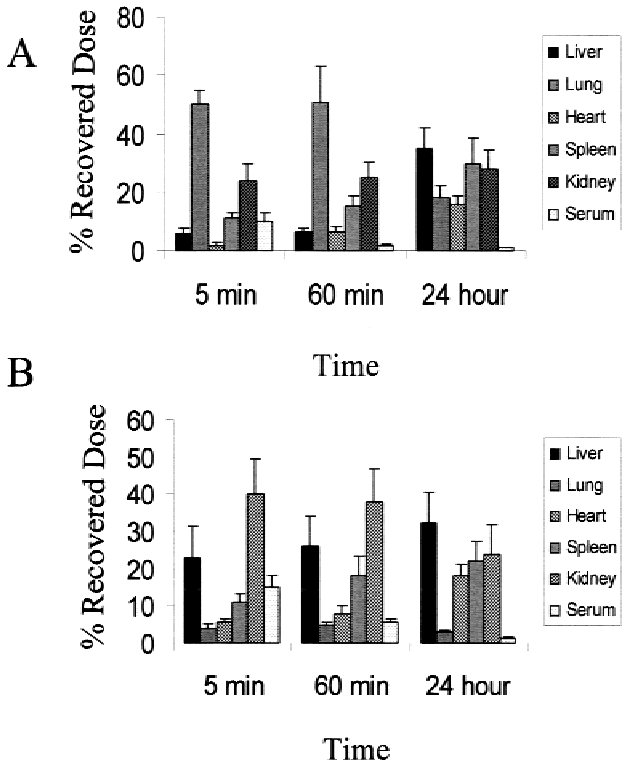

Fig. 2. Tissue distribution profiles of DNA after intravenous administration of the TerplexDNA [^{32}P]DNA (plasmid DNA) (A) and naked plasmid [^{32}P]DNA (B). At 5 min, 60 min, and 24 h after DNA injection, the whole body of the animals was perfused with saline and their organs were removed. The weighed amounts of tissue samples were solubilized in Soluable™, mixed with counting medium (Hionic-Fluor™), and counted for its radioactivity using a β-counter.

In Vivo Gene Expression After Systemic Administration

Luciferase expression plasmid (pCMV-Luc) was tested and characterized both *in vitro* and *in vivo* previously (7,8). In this study, the same plasmid was used as a reporter gene to evaluate the TerplexDNA system mediated gene transfer *in vivo* by analysis of the luciferase activity. After injection of the TerplexDNA/ pCMV-Luc complexes via mouse tail-vein, the significant luciferase activities were detected in the liver, lung, heart, spleen, and kidney at various time points (Fig. 3). However, the luciferase gene expression with naked plasmid pCMV-Luc alone was very low in these major organs (Fig. 4). Liver is the highest luciferase gene expression organ both in the TerplexDNA/pCMV-Luc mediated gene delivery and naked plasmid DNA (pCMV-Luc) transfection. The luciferase gene expression with the TerplexDNA system reached a peak at day 3 and then gradually decreased whereas luciferase gene expression with naked DNA reached a peak at 16 h after injection. The luciferase gene expression in major organs with the TerplexDNA system mediated gene transfer was detected for 35 days after systemic administration whereas naked plasmid DNA mediated luciferase gene expression in major organs was detected only for 7 days after systemic administration. *In vivo* gene expression studies indicated that after systemic injection, the TerplexDNA system showed the significant amount of gene expression in major organs with prolonged duration of gene expression and delayed gene expression peak compared to the gene expression in major organs in naked plasmid DNA set.

DISCUSSION

This work describes the pharmacokinetics, tissue distribution, and *in vivo* gene expression of the plasmid DNA component after intravenous administration of the TerplexDNA system as part of the preclinical development of the TerplexDNA delivery vector. The fate and function of the plasmid DNA or synthetic gene carrier/DNA complex *in vivo* can be affected by many biologic factors such as cell surface molecules and chemistry, circulatory pattern, and rapid elimination from blood or interstitial space due to the enzymatic degradation or organ clearance. In our study, LDL was used as a ligand moiety, which complexes with stearyl-PLL via hydrophobic interactions. By making apolipoprotein portion of LDL available for the LDL receptor binding by carefully controlling optimal ratio between the components, the TerplexDNA system has shown efficient transfection activity *in vitro* (5–7,9) and *in vivo* (8). An atomic force microscopy study has shown that when the stearyl-PLL and LDL were added to the plasmid DNA to form a TerplexDNA system, the plasmid DNA was condensed to 100 nm along the long axis (5). The zeta-potential was about +2 mV at a 1:1:1 weight ratio of DNA: LDL: stearyl-PLL, which resulted in the best transfection efficiency among those tested (5). Based on *in vitro* data, we expended our previous work and investigated the TerplexDNA system in pharmacokinetics, biodistribution, and *in vivo* systemic gene expression activities for further applications.

Our pharmacokinetic data were analyzed based on total radioactivity in the blood at each time point, which showed that the TerplexDNA remained in the circulation for a longer period of time and distributed into the body more extensively compared to naked plasmid DNA (Table I). It is known that naked plasmid DNA is rapidly cleared from blood by both enzymatic degradation and organ uptake within minutes if there is no gene carrier protecting them. Naturally, LDL is a normal particle in human circulation. Incorporation of LDL may delay the TerplexDNA system from being cleared from the circulation by escaping from extracellular opsonin recognition and phagocytic cell attack (Fig. 1). The various cationic polymers and lipid have been used in many gene delivery studies for protecting DNA from enzymatic degradation, modifying the size, charge, and surface characteristics of DNA, and controlling the fate of DNA in the body. However, there are few reports describing a novel gene carrier design for protection of the gene carrier/DNA complex from an extracellular element attack.

A high volume of distribution was obtained from the TerplexDNA administration (2.61 L/kg), suggesting that the TerplexDNA system has a high affinity to the tissues, possibly owing to the use of LDL as a ligand for receptor-mediated endocytosis. Molecular conjugates employing the ligands have been used to direct the gene or gene/carrier complex to the tissue displaying the specific receptors and enhances the cellular bioavailability of the gene by receptor mediated endocytosis (10–12). Given the fact that LDL receptors are prevalent in specific organs in the body, the TerplexDNA system could be an efficient systemic gene delivery vector by enhancing the organ disposition and cellular internalization of the gene.

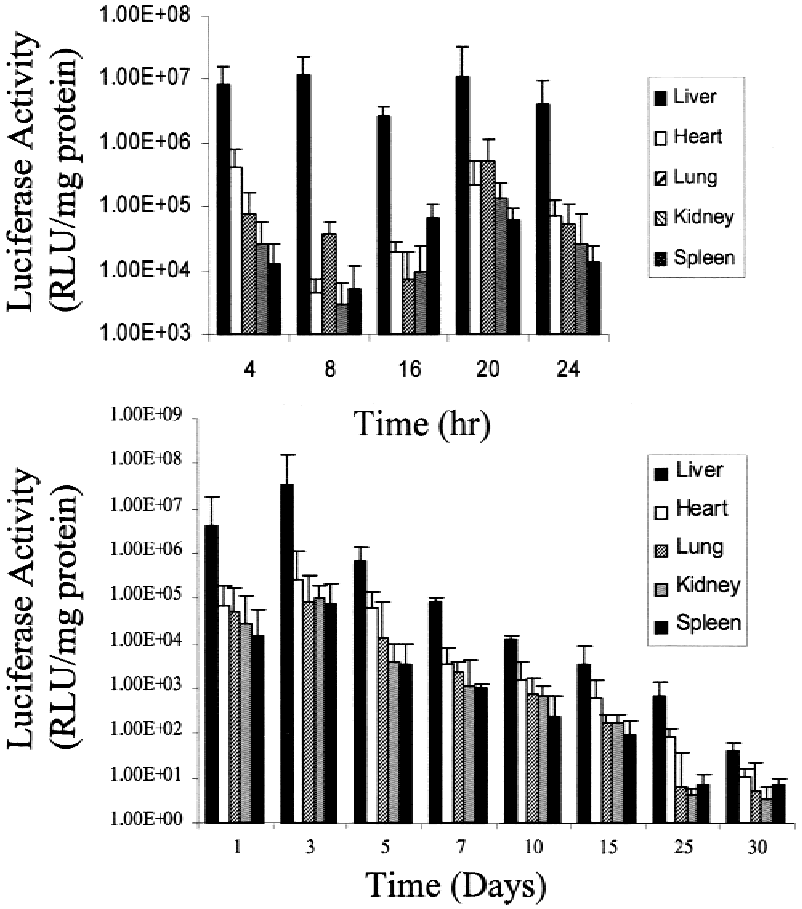

Fig. 3. Time-dependent luciferase gene expression in major organs after the TerplexDNA gene carrier mediated gene transfer via mouse tail-vein injection. (Top panel) early gene expression time course and (bottom panel) later gene expression time course.

After intravenous administration of the TerplexDNA system, the primary DNA disposition organ was the lung during first hour after injection whereas the amount of DNA in the liver increased gradually with time and became a major disposition organ in 24 hs after injection. Several mechanisms have been proposed for the distribution of the complexes to the lung during early time after intravenous administration (13). One mechanism proposed is that the pulmonary capillaries act as a "sieve" for the DNA/polymer complexes. Because the pulmonary microvasculature is the first capillary

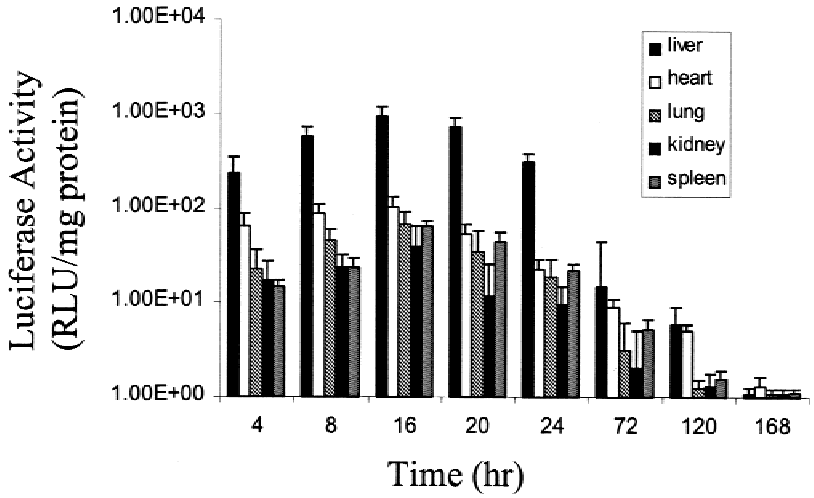

Fig. 4. Time-dependent luciferase gene expression in major organs after naked plasmid DNA mediated gene transfer via mouse tail-vein injection.

bed encountered by intravenously injected complex, aggregation of the 100–200 nm polymeric complexes in the blood could lead to passive targeting to the lung by this mechanism (14). It is known that particulates with a diameter >5- to 7-μm lodge in the pulmonary capillary beds (15,16). Therefore, unless significant aggregation of the complexes occurs in the blood immediately upon contact, the mechanism of physical entrapment remains uncertain.

The TerplexDNA in each organ appeared to experience the redistribution process over time. DNA in the lung continued to decrease over time and only a small portion of DNA was left in the lung 24 h after injection, and eventually the liver became the major DNA disposition organ. The decrease of DNA portion in the lung may be explained by the dissociation of DNA from the complex by interpolyionic exchange reaction between the large excess amounts of negatively charged blood components and the DNA complexed with positively charged polymer (17). The dissociation of DNA from the complex by polyanion (heparin sulfate) *in vitro* was confirmed in our electrophoresis study (data not shown). The dissociated DNA or small DNA aggregates in the lung capillaries can be carried away by blood flow, resulting in the change of DNA biodistribution profiles.

Gene therapy requires both effective gene transfer and effective gene expression in desired cells. The pharmacokinetic and biodistribution studies of the TerplexDNA system showed the fate of the TerplexDNA complexes after they were administrated in the body, while gene expression studies showed the gene transfer efficiency of the TerplexDNA system. The luciferase gene is one of the most widely used reporter genes for quantitative analysis of gene expression and for gene transfection efficiency in gene therapy research. The luciferase reporter gene system is a simple, sensitive, specific, and quantitative method (18). There is no endogenous luciferase activity in human body compared to that of β-galactosidase. According to the pharmacokinetic and biodistribution data above, we analyzed the TerplexDNA mediated gene expression in major organs over the time-course to compare the gene expression mediated by the naked plasmid DNA in both gene expression level and gene expression duration.

In the present studies, luciferase activities was mainly detected in the liver even though they were also detected in other major organs (Figs. 3 and 4) after the TerplexDNA complex or naked plasmid DNA was administrated via mouse tail-vein injection. Luciferase gene expression by the TerplexDNA mediated gene transfer is approximately two orders higher than that by naked plasmid DNA alone in all tested time points (Figs. 3 and 4), whereas the reporter gene expression mediated by PLL or PLL-stearyl gene carriers showed no significant improvement compared to naked plasmid DNA alone (data not shown). The peaks of the luciferase gene expression in the tested organs appeared at day 3 after the administration compared to the peak at 16 hours after naked plasmid DNA administration. Using the TerplexDNA system, the luciferase activity in major organs remained to be detected at day 25 after injection (Fig. 3), while it was hard to be detected at day 7 after naked plasmid injection (Fig. 4). The data from Figs. 3 and 4 indicated that the TerplexDNA system significantly enhanced *in vivo* gene transfer and gene expression with prolonged gene expression duration. The longer retention of the gene in the body with the TerplexDNA administration (MRT, 7.84 h) compared with naked DNA administration (MRT, 2.54 h) could be the reason for the prolonged gene expression. In an *in vivo* situation, it is difficult to make DNA available to the cell surface since the transfection is performed in a physiologic and dynamic environment. There are two classes of gene transfer barriers that inhibit gene carrier mediated gene delivery and gene expression: extracellular barriers and intracellular barriers (19). The four major main extracellular barriers are opsonins, phagocytes, extracellular matrices, and degradative enzymes. LDL is a normal component present in circulation. LDL may facilitate the TerplexDNA system escape gene from microphage cells and opsonin's attack in circulation, thus prolong the circulation time in the bloodstream and increase the exposure time to their receptor. However, the exact reason that the TerplexDNA system enhanced gene transfer *in vivo* is still unknown.

There is a discrepancy between the data from pharmacokinetics/biodistribution and the gene expression of the TerplexDNA system. In pharmacokinetics/biodistribution studies, the lung was the first major organ of the TerplexDNA complex accumulation in early time points (Fig. 2). However, in gene expression studies, the lung was the second or third major organ while the liver, rich in LDL receptors, became the first major organ (Fig. 3). These observations may suggest that the TerplexDNA system-mediated gene transfer may be LDL receptor specific.

TerplexDNA gene carrier is a stable and self-assembled novel lipopolymeric gene carrier system. The advantage in transfection efficiency may be related to the unique properties of the Terplex "supramolecules." This novel delivery system has the potential for gene therapy applications. Further work will be conducted to study the interaction of the TerplexDNA system with the circulation system in the body.

ACKNOWLEDGMENTS

We thank Dr. Joseph Balthasar for valuable discussions, Qifu Wang for technique support, NIH (HL-65477), and Expression Genetics for the financial support.

REFERENCES

1. T. Friedmann. *The Development of Human Gene Therapy.* Cold Spring Harbor Laboratory Press, San Diego, CA, 1999.
2. W. F. Anderson. Human gene therapy. *Nature* **392**:25–30 (1998).
3. H. I. Miller. Gene therapy on trial. *Science* **287**:591–592 (2000).
4. P. L. Felgner, O. Zelphatl, and X. W. Liang. Advances in synthetic gene-delivery system technology. In T. Friedmann (ed.), *The Development of Human Gene Therapy,* Cold Spring Harbor Laboratory Press, San Diego, CA, 1999 pp. 241–260.
5. J. S. Kim, A. Maruyama, T. Akaike, and S. W. Kim. A new non-viral DNA delivery vector: the TerplexDNA system. *J. Control. Release* **53**:175–182. (1998a).
6. J. S. Kim, A. Maruyama, T. Akaike, and S. W. Kim. Terplex DNA delivery system as a gene carrier. *Pharm. Res.* **15**:116–121 (1998b).
7. L. Yu, M. Nielsen, and S. W. Kim. TerplexDNA gene carrier system targeting to artery wall cells. *J. Control. Release* **72**:179–189 (2001).
8. D. G. Affleck, L. Yu, D. A. Bull, S. H. Bailey, and S. W. Kim. Augmentation of myocardial transfection using TerplexDNA: a novel gene delivery system. *Gene Ther.* **83**:349–353 (2001).
9. J. S. Kim, A. Maruyama, T. Akaike, and S. W. Kim. *In vitro* gene expression on smooth muscle cells using a TerplexDNA delivery system. *J. Control. Release* **47**:51–59 (1997).
10. E. Wanger, M. Cotten, F. Rolland, and M. L. Birnstiel. Transferrin-polycation-DNA complexes: The effect of polycations on the

structure of the complex and DNA delivery to cells. *Proc. Natl. Acad. Sci. USA* **88**:4255–4259 (1991).
11. G. Y. Wu and C. H. Wu. Receptor-mediated gene delivery and expression *in vivo*. *J. Biol. Chem.* **263**:14621–14624 (1988).
12. S. Gottschalk, R. Cristiano, L. Smith, and S. Woo. Folate-mediated gene delivery and expression *in vitro*. *Gene Ther.* **1**:185–191 (1994).
13. F. D. Ledley. Nonviral gene therapy: The promise of genes as pharmaceutical products. *Hum. Gene Ther*. **6**:1129–1144 (1995).
14. K. A. Mislick and J. D. Baldeschwieler. Evidence for the role of proteoglycans in cation-mediated gene transfer. *Proc. Natl. Acad. Sci. USA* **93**:12349–12354 (1996).
15. L. C. Mounkes, W. Zhong. G. Dipres-Palacin, T. D. Heath, and R. J. Debs. Proteoglycans mediate cationic liposome-DNA complex-based gene delivery *in vitro* and *in vivo*. *J. Biol. Chem.* **273**: 26164–26170 (1998).
16. R. Timpl. Proteoglycans of basement membranes. *Experientia* **49**:417–428 (1993).
17. S. Katayose and K. Kataoka. Water-soluble polyion complex associates of DNA and poly(ethylene glycol)-poly(L-lysine) block copolymer. *Bioconjugate Chem.* **8**:702–707 (1997).
18. D. Gal, L. Weir, G. Leclerc, J. G. Pickering, J. Hogan, and J. M. Isner. Direct myocardial transfection in two animal models. *Lab. Invest.* **68**:18–24 (1993).
19. L. Huang and E. Viroonchatapan. Part I: Introduction in nonviral vectors for gene therapy. In L. Huang, M.C. Hung, and E. Wanger (eds.), *Nonviral Gene Vectors.* Academic Press, London, 1999 pp 1–32.