Pharmacokinetics of Gene Delivery in Cells

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1 Introduction

1.1 Intracellular Barriers to Gene Delivery

Since the first report, in the 1980s, of gene delivery with a cationic liposome, numerous attempts have been made to improve non-viral gene vectors. It has gradually become clear that transgene expression can be interrupted by several intracellular barriers (Fig. 1). The cellular association of naked DNA molecules is very poor, since negative charges on both the cell surface and the DNA molecules interrupt contact with each other via electrostatic interactions. Thus, in order to enhance cellular association, DNA was condensed with cationic polymers (Oupicky et al. 2000; Brown et al. 2001) and cationic liposomes (Li et al. 1999; Tranchant et al. 2004) that neutralize the effect of the negative charge. A cationic vector enhances cell-surface binding through interactions with the negative constituents of the cell surface (e.g. heparan sulfate proteoglycans) or through selective binding to specific receptors, resulting in a strong transgene expression. This method of condensation also enables targeting of the cells by modulating different ligands to the surfaces of the gene vectors (Table 1).

Currently, several ligands are used for recognition by their specific receptors and uptake via cellular receptor-mediated endocytosis. In this pathway, however, lysosomal degradation presents a second barrier for gene vectors. Before the endosomal membrane fuses with the lysosome, the gene vectors must be released into the cytosol. The importance of endosomal escape is clearly evidenced by the drastically enhanced transfection efficiency in the presence of lysosomotropic reagents, such as chloroquine (Erbacher et al. 1996), which accumulate in the acidic lysosome and destabilize the membrane by causing it to swell. Various devices, such as pH-sensitive fusiogenic lipids (Farhood et al. 1995; Hui et al. 1996; Harashima et al. 2001), polycations that have proton sponge ability (Boussif et al. 1995; Kichler et al. 2001), and pH-sensitive

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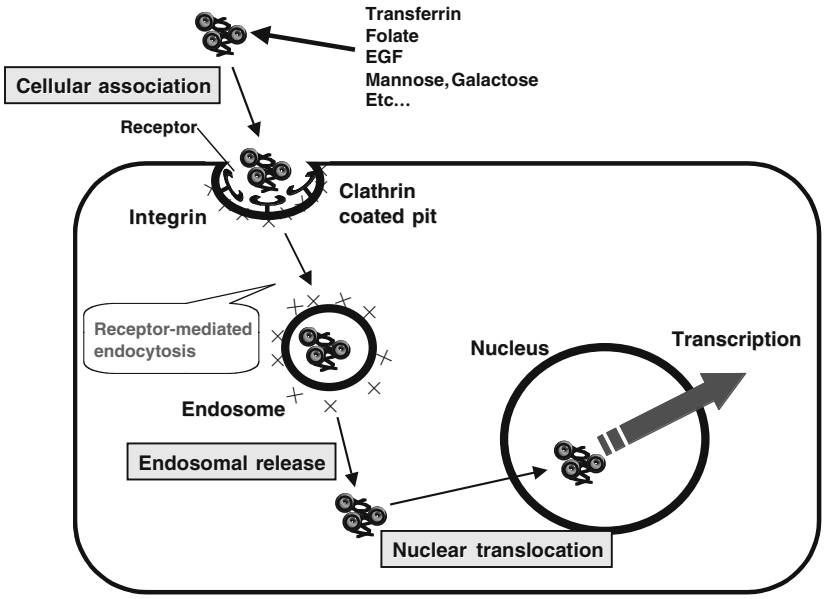

FIG. 1. The commonly accepted intracellular trafficking pathways of non-viral gene vectors. It is generally accepted that endocytosis is a major pathway for cellular entry. After internalization, the gene vector must be released to the cytosol to escape lysosomal degradation. Finally, the vector must be translocated through the nuclear membrane for transcription. In order to overcome these barriers, it is necessary to develop functional devices and combine them in such a way so as to exhibit all of the functions at the appropriate site and with realistic timing

TABLE 1. Various functional devices for overcoming cellular barriers

Barriers	Plasma membrane	Endosomal membrane	Nuclear membrane
Functional devices	Specific ligands (transferrin, insulin, EGF, etc.)	pH-sensitive peptides (GALA, HA2, etc.)	NLS peptides (mu, protamine, etc.)
	PTD peptides (octaarginine, Tat, etc.)	pH sensitive lipids (CHEMS, DOPE, etc).	PTD peptides (octaarginine)
	Cationic lipids (DOTMA, DOTAP, DOSPA, etc.)	Proton sponge polymers (PEI) PTD peptides (octaarginine)	

membrane lytic peptides (Wagner et al. 1992; Plank et al. 1994; Wagner 1998) can also aid in endosomal escape (Table 1), as described below.

The final barrier to the intracellular trafficking of DNA is the nuclear membrane. Since the size threshold for freely passing through the nuclear pore complex is 50 kDa, commonly used plasmid DNA (pDNA) is too large to pass, unassisted, through the nuclear-pore complexes (Lycke et al. 1988; Hagstrom et al. 1997; Sodeik et al. 1997;

Sebestyen et al. 1998; Branden et al. 1999; Ludtke et al. 1999; Wilson et al. 1999). Therefore, plasmid DNA is thought to enter into the nucleus when the nuclear membrane structure disappears during the M-phase of mitosis (Wilke et al. 1996; Marenzi et al. 1999; Mortimer et al. 1999; Tseng et al. 1999). In fact, the percent of plasmid DNA, reaching the nucleus has been reported to be less than 1% of the total cytoplasmically microinjected DNA (Pollard et al. 1998). Therefore, in order to improve entry into the nucleus, nuclear localization signal peptides (NLS) are attached either to the plasmid DNA itself (Sebestyen et al. 1998; Zanta et al. 1999; Nagasaki et al. 2003; Tanimoto et al. 2003) or to counter-polycations (Chan and Jans 1999; Chan et al. 2000). Various reports on the use of NLS peptides as polycations have recently been published (Li and Huang 1997; Li et al. 1998; Murray et al. 2001; Rittner et al. 2002; Tagawa et al. 2002; Keller et al. 2003; Rudolph et al. 2003). Although these approaches succeed in enhancing transgene expression, the technology for delivering plasmid DNA to the nucleus in non-dividing cells needs further development.

1.2 Topics in This Chapter

In order to develop efficient gene vectors, new devices to overcome the intracellular barriers discussed above must be designed. Furthermore, these devices should be assembled into one vector, taking the topology of these devices into consideration so that their functions are utilized at the appropriate time and position. In this chapter, we first introduce the concept of Programmed Packaging; a novel packaging concept for a non-viral gene delivery system. We then describe our recent development of a Multifunctional Envelope-type Nano-Device (MEND) for use in either the endocytic or non-endocytic pathways.

In order to optimize gene delivery, it is necessary to quantitatively evaluate the function of the devices in cells. However, quantitative information regarding, e.g., endosomal release and nuclear translocation, are lacking. We recently succeeded in addressing this problem by developing a three-dimensional quantitative analysis for the intracellular trafficking of plasmid DNA delivered with non-viral vectors. The importance of the pharmacokinetic considerations of intracellular trafficking of DNA based on this methodology are discussed below.

2 A New Packaging Concept for Non-viral Vectors for Efficient Gene Delivery: Programmed Packaging

Various non-viral systems have been developed to date and can be classified as follows. The first generation consists of DNA/cation complexes, in which DNA is simply mixed with positively charged molecules, such as cationic polymers (Kawai et al. 1984; Boussif et al. 1995; Tang et al. 1997), cationic detergents (Blessing et al. 1998) and cationic liposomes (Felgner et al. 1987; Felgner et al. 1994; Sorgi et al. 1997; Gao et al. 1996), to form complexes via electrostatic interactions. For example, a complex consisting of DNA and polyethylenimine (PEI), which is known to function as a proton sponge, had significantly high transgene activity in vitro (Boussif et al. 1995). However, such complexes easily aggregate with serum proteins and/or red blood cells in blood and accumulate in the lung capillary (Lee et al. 1996). This problem was overcome by a

second generation of gene delivery systems, in which plasmid DNA encapsulated in lipid vesicles and/or a PEI/DNA complex were covered with polyethylene glycol (PEG) in order to avoid interactions with blood components (Lee et al. 1996; Ogris et al. 1999; Wheeler et al. 1999). Furthermore, these second-generation gene delivery systems can be equipped with specific targeting ligands, such as transferrin and folate at the PEG terminal (Lee et al. 1996; Ogris et al. 1999; Kircheis et al. 2001), which prolongs the circulation time in the blood (Wheeler et al. 1999) and allows selective tumor targeting (Ogris et al. 1999; Kircheis et al. 2001; Tam et al. 2000). However, while these vectors can reach the target cells in vivo, they cannot efficiently escape from the endosomes to the nucleus (Wheeler et al. 1999; Kircheis et al. 2001; Tam et al. 2000).

For efficient gene delivery, the advantages of both generations need to be integrated into a packaged delivery system. Moreover, intracellular trafficking of the delivery system should be controllable with the devices introduced. However, it is not easy to assemble such devices in a manner in which each device can function appropriately. Therefore, we have proposed a new concept, Programmed Packaging, to represent the next (third) generation of gene delivery systems. In Programmed Packaging, functional devices are able to overcome the barriers posed by cells at the correct time and at the appropriate place in order to control the pharmacokinetics (PK) and pharmacodynamics (PD) of a gene delivery system. Programmed Packaging consists of three features: (1) a strategy to overcome the barriers; (2) the design of functional devices to overcome the barriers and their assignments; and (3) the assembly of these components into a nano structure. We recently developed a novel non-viral gene delivery system, which we named a "Multifunctional Envelope-type Nano Device (MEND)" (Kamiya et al. 2003; Kogure et al. 2004). The ideal scheme of MEND is shown in Fig. 2. The complete system consists of a condensed DNA core and a lipid envelope structure equipped with various functional devices. The condensed core has several advantages, such as the protection of DNA from DNase, size control and enhanced packaging efficiency. Furthermore, the core-envelope structure confers the ability to control the topology of the functional devices. The NLS should be located on the surface of the core particles for targeting the nucleus and the protein transduction domain (PTD) peptide should be located on the envelope in order to control the entrance pathway. In addition, a pH-sensitive fusiogenic peptide should be incorporated on the envelope for enhanced endosomal escape (Kakudo et al. 2004). Fourth-generation gene delivery systems might thus be represented by an "artificial-intelligent nano-device (AIND)." The AIND would be equipped with arms to kill the virus and a system to convert chemical energy to kinetic energy; which can be denoted a "nano-machine."

3 Control of the Intracellular Trafficking of Genes

3.1 Receptor-mediated Endocytosis: Enhanced Endosomal Escape with Cholesterol GALA Peptide

It is important to select the appropriate entrance route in order to optimize intracellular trafficking, since the intracellular fate of internalized DNA with the carrier system is determined principally by a constitutive map of intracellular trafficking of

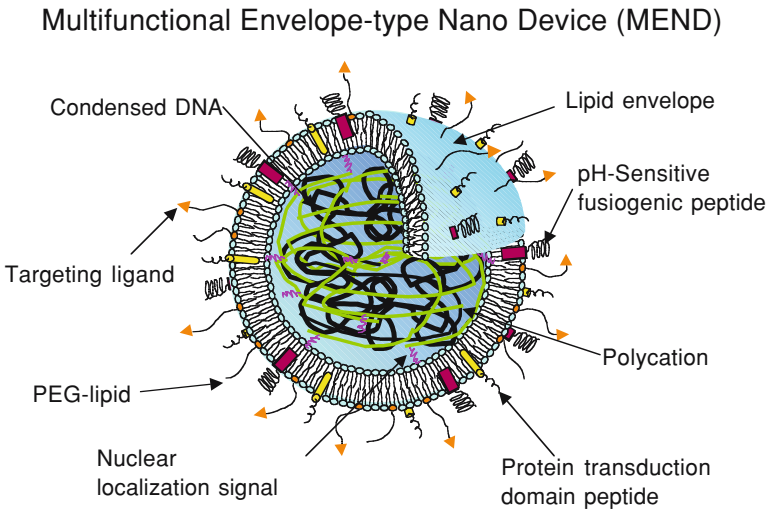

FIG. 2. Ideal multifunctional envelope-type nano device (MEND). MEND was designed to have multifunctional devices, such as specific ligands, for selective cellular delivery, PEG for prolonged circulation in the blood, a pH-sensitive fusiogenic peptide for endosomal escape after receptor-mediated endoscytosis, protein transduction domains (PTDs) for efficient cellular uptake, and a nuclear localization signal for efficient nuclear translocation. MEND also has a specific mechanism for enhancing intranuclear transcriptional efficiency, thus maximizing the efficacy of gene expression

the endogenous substance. In general, conventional endosomes are able to fuse with lysosomes, where DNA/proteins are degraded. Therefore, it is essential for the device to escape from endosomes/lysosomes prior to degradation. The utilization of receptor-mediated endocytosis would be expected to function as a pathway for the intracellular delivery of anticancer drugs in cancer chemotherapy as well as nucleic acids in gene therapy (Harashima et al. 2001). However, once endocytosed, the control of intracellular trafficking is difficult, since it is under the regulation of the cell. Therefore, in order to optimize intracellular trafficking after receptor-mediated endocytosis to a target organelle, such as the cytosol and the nucleus, artificial sorting devices are required (Kamiya et al. 2001). Liposomes are one of the most promising systems for selective cellular targeting by introducing specific ligands for cell-surface receptors.

We have succeeded in elucidating the intracellular fate of transferrin-modified liposomes and in altering it by introducing the pH-sensitive fusiogenic peptide, GALA (WEAALAEALAEALAEHLAEALAEALEALAA). Transferrins that are chemically attached to the liposomal surface (Tf-L) are internalized via receptor-mediated endocytosis more slowly than unmodified transferrins (Kakudo et al. 2004). In contrast to the recyclable nature of transferrin, liposome-attached transferrins, together with encapsulated rhodamines, were retained in the vesicular compartments.

The intracellular fate of Tf-containing Chol-GALA was analyzed by confocal laser scanning microscopy (CLSM) using K562 cells. As shown in Fig. 3c, a dramatic cytoso-

lic release of encapsulated sulfo-rhodamine (S-Rh) was observed, while S-Rh in Tf-L with encapsulated GALA was not released (Fig. 3b). These results suggest that the endosomal release of S-Rh proceeded quite efficiently with the aid of the GALA present on the liposome surface. It is noteworthy that, in the case of GALA-encapsulated Tf-L, Tf was entrapped in the endosomes/lysosomes, while in the case of Chol-GALA, Tf was recycled and remained on the plasma membranes (Fig. 3c, Fig. 4). Therefore,

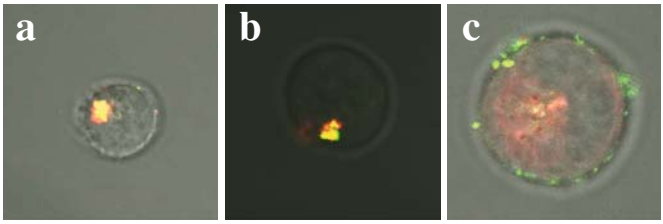

FIG. 3. **a** Confocal laser scanning microscopy (CLSM) of Tf-L, **b** Tf-L with GALA, and **c** Tf-L with Chol-GALA. FITC-labeled Tf-modified liposomes encapsulating sulforhodamine as an aqueous-phase marker were administered to K562 cells and analyzed by CLSM after 18 h

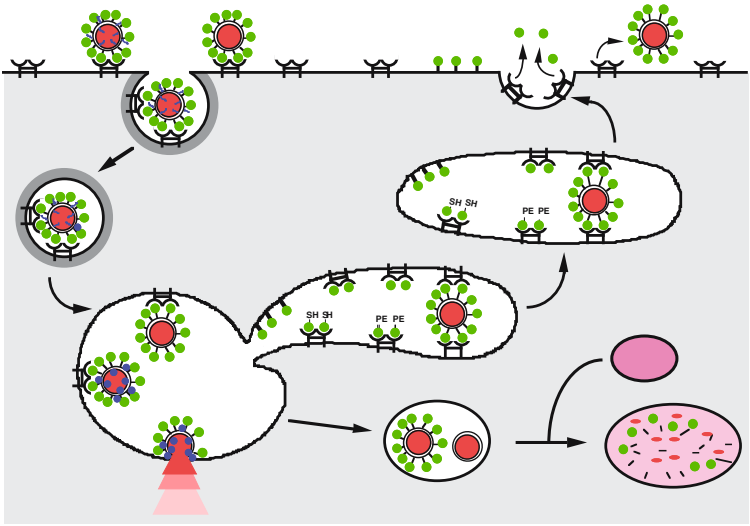

FIG. 4. Scheme for the intracellular trafficking of Tf-L with Chol-GALA. Tf-L were internalized by Tf-receptor-mediated endocytosis. In an endosome, the pH decreases from 7.4 to ~6.0, which induces a conformational change in Chol-GALA that enhances membrane fusion between the endosomal liposomal membranes. The aqueous marker is released to the cytosol and liposomally attached Tf is distributed to the endosomal membrane. After recycling of the endosome, the Tf remains on the plasma membrane, since the Tf originally introduced to the liposomal membrane is transferred to the endosomal membrane by fusion via Chol-GALA, then fused with the plasma membrane

Chol-GALA significantly affected the intracellular fate of Tf-L. These results suggest that the topology of GALA, namely, its surface disposition on the liposomal membrane, is a critical factor in these effects.

GALA was designed to preferentially interact with neutral bilayers at a low pH, considering factors such as the hydrophobicity of the residues, the conformational preference of the amino acids, the length of the peptides, and the topology of the residues on the peptide (Subbarao et al. 1987; Nir and Neiva 2000). The peptide contains 30 amino acids, with a repeated sequence of Glu-Ala-Leu-Ala (GALA). When the pH of the solution is decreased, protonation of the Glu residues weakens the repulsive forces, thus inducing the formation of a hydrophobic helical structure. In the presence of lipid membranes, the peptide, readily fuses with the lipid membranes (Goormaghtigh et al. 1991; Parente et al. 1988; Parente et al. 1990; Duzgunes and Nir 1999). In vitro energy transfer and dynamic light scattering experiments indicated that endosomal escape of the encapsulates in Tf-L equipped with Chol-GALA could be attributed to the pH-dependent membrane fusion (Kakudo et al. 2004).

This system has great potential for gene delivery applications by delivering encapsulated, condensed plasmid DNA to tissue tumors, where transferrin liposomes would be expected to be internalized by receptor-mediated endocytosis with the aid of Chol-GALA by membrane fusion between liposomes and the endosome (Fig. 4). The condensed plasmid DNA could then be effectively released into the cytosol.

3.2 Non-endocytic Routes for Gene Delivery

3.2.1 Protein Transduction Domains

Since endocytosis acts as a significant intracellular barrier, as explained above, it would be more advantageous to bypass the endocytic pathway and somehow achieve the cytosolic delivery of genes using other pathways. The non-endocytic delivery of genes can be achieved by microinjection, electroporation and cell permeabilization; however, these techniques are highly invasive and cannot be used for in vivo gene delivery. One interesting, non-invasive technique is the use of different peptides and proteins with membrane-translocating properties (Schwarze et al. 2000; Lindgren et al. 2000). Several proteins that possess the ability to enter cells have been identified, including the HIV-1 TAT protein and the *Drosophila* Antennapedia (Antp) transcription factor. Surprisingly, internalization of these PTDs is largely due to relatively short peptide sequences, termed membrane-translocationable signals (MTSs). For example, the minimal signal peptide required for intracellular translocation consists of the basic residues 48-60 of the TAT protein (Futaki 2002). The mechanism of uptake of these peptides is currently controversial; there are arguments for and against endocytic uptake (Polyakov et al. 2000; Torchilin et al. 2001; Terrone et al. 2003; Richard et al. 2003; Ferrari at al. 2003; Wadia et al. 2004). However, uptake appears to be different from classical clathrin-dependent endocytosis. The sequence of TAT-(48-60), which is critical for the translocation, is highly basic, containing six arginines and two lysines in 13 amino acid residues. The arginine-substituted analog of TAT was found to be internalized into cells as efficiently as the TAT-(48-60) peptide, suggesting the importance of arginine residues in the translocation process (Futaki et al. 2001a). Peptides consisting of only arginine residues were also internalized and the optimum

number of arginine residues for efficient internalization was shown to be approximately eight (Futaki et al. 2001a). Therefore, we became interested in the R8 peptide for use in the non-endocytic delivery of genes.

First, the mechanism by which the R8 peptide is internalized was investigated. CLSM of fixed cells showed that the R8 peptide was internalized equally well at 37°C and 4°C and was transported to the cytosol and nucleus (Fig. 5a). Similarly, different endocytosis inhibitors, including hypertonic treatment, *N*-ethyl maleimide and nystatin, had no effect on internalization of the peptide (Khalil et al. 2004). These results were confirmed using flow cytometry of live cells. R8 was then used to condense plasmid DNA, and a positively charged complex of ~100 nm in diameter was obtained. Again, using CLSM, the R8/DNA complexes were shown to be internalized into the cytosol of cells (Khalil et al. 2004). However, CLSM of fixed cells under the same conditions used in the case of R8 peptide showed that internalization of the R8/DNA complexes was significantly inhibited (~90%) at low temperature (Fig. 5b) or by treatment with a hypertonic agent that specifically inhibits clathrin-mediated endocytosis. Furthermore, it was observed that the complexes were trapped in endosomes and lysosomes, thus decreasing the effectiveness of the vector (Akita et al. 2004). Therefore, the uptake of R8 peptide and R8/DNA complexes under similar conditions was compared, and significant differences in the uptake process in the presence of endocytosis inhibitors were found, indicating that the mechanism of internalization of the R8 peptide is not exactly the same as that for its complexes with DNA. While the R8/DNA complexes were taken up by clathrin-mediated endocytosis, the R8 peptide seemed to use different uptake pathways. Therefore, by direct complexation with DNA, the peptide fails to internalize the DNA away from endocytosis, where the endosomal

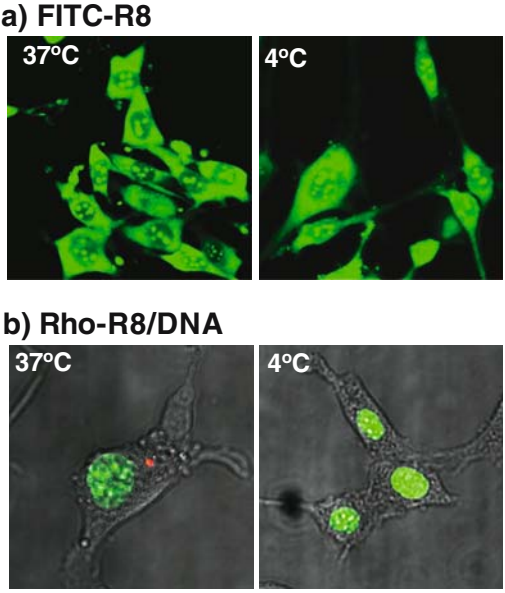

Fig. 5a,b. Internalization mechanisms of R8 peptide and R8/DNA complexes. NIH3T3 cells were incubated with **a** FITC-labeled R8 (2 μM) or **b** rhodamine-labeled plasmid DNA complexed with R8 (2.5 μg/ml) for 1 h at 37°C or at 4°C followed by fixation and observation by CFLM. In the case of R8/DNA complexes, the nucleus was stained with Syto-24 green fluorescence

barrier still exits. Whether the peptide is taken up through penetration or through endocytic pathways other than classical endocytosis is not clear.

3.2.2 Stearylated Octaarginine

One possibility for explaining the endocytic delivery of R8/DNA complexes is that the peptide is in contact with the DNA, and as a result, is not free to interact with cellular membranes. Therefore, R8-modified liposomes (R8-Lip); lipid vesicles modified with free R8 peptide that can carry genes or proteins in their cores, were prepared. In order to present the R8 peptide on the surface of the liposomes, it was necessary to add an anchor to the peptide to be embedded in the lipid of the vesicle, thus leaving the free peptide on the surface. Therefore, a stearylated-R8 peptide (STR-R8) was used in which the stearyl moiety would be expected to be anchored in the lipid membrane of the liposomes. Surprisingly, complexes formed by simply mixing the STR-R8 peptide and plasmid DNA showed improved transfection activities compared to R8/DNA complexes (Futaki et al. 2001b). This result was confirmed by demonstrating that STR-R8/DNA complexes showed transfection activities at least three orders of magnitudes higher than those of the R8/DNA complexes (Fig. 6a). The mechanism of the improved transfection activity of the STR-R8 peptide was subsequently investigated. Cellular uptake of DNA complexed with STR-R8 was significantly higher than that of DNA complexed with R8 (Fig. 6b). Furthermore, the internalized DNA was partially localized in the nucleus in the case of STR-R8, but not in case of R8 (Fig. 6b). The internalization of both complexes was inhibited in the presence of endocytosis inhibitors, indicating endocytic delivery (Khalil et al. 2004). In order to explain the improved nuclear delivery in the case of STR-R8, cells were incubated with complexes formed from differently labeled DNA and peptides. In the case of the R8/DNA complexes, a high degree of co-localization was found between the peptide and the DNA, indicating that the DNA is present in cells in the complexed state (Fig. 6c). In contrast, free DNA was found intracellularly in the case of STR-R8, indicating that the DNA is released more easily from the complexes. Furthermore, using our novel quantitative intracellular trafficking technique, described below, it was observed that the DNA was able to escape from endosomes in the case of STR-R8 only (Akita et al. 2004). These collective findings suggest that STR-R8 has the ability to disrupt endosomes by itself, releasing free DNA to the cytosol, which may be more easily delivered to the nucleus. Using flow cytometry to differentiate between internalized and surface-bound DNA, surface association of DNA in the case of STR-R8 was found to be much higher than that in the case of R8 (Khalil et al. 2004). This improvement was confirmed by atomic force microscopy (AFM), which showed that STR-R8, but not R8, had the ability to condense the DNA completely into stable cores (Fig. 6d). Aided by both electrostatic and hydrophobic interactions, the STR-R8/DNA complexes would be highly adsorbed to cell membranes and subsequently highly internalized.

3.2.3 Octaarginine-Modified Liposomes

Since our data indicated that direct complexation between R8 and DNA allowed endocytic delivery of the complexes, we focused on our main objective, i.e., effective cellular internalization of genes in the absence of typical endocytosis. R8-Lip were prepared by mixing the lipids of the liposomes with STR-R8. R8-Lip, containing 5

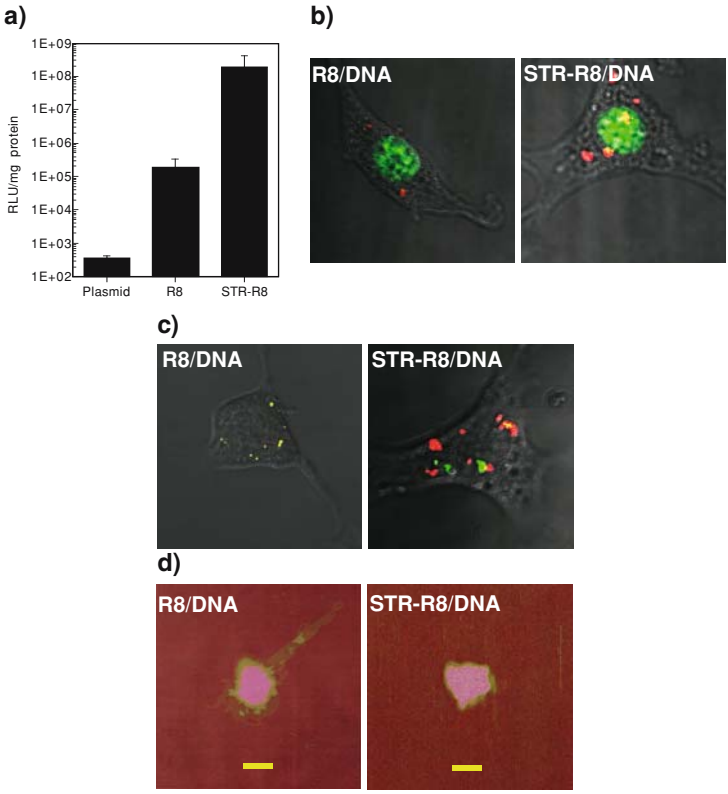

FIG. 6. **a** Transfection activities of R8 and STR-R8 peptides. NIH3T3 cells were transfected with plasmid DNA coding for the luciferase reporter gene (2.5 μg/ml) complexed with R8 or STR-R8 (cation-anion charge ratio 2:1) for a total of 48 h. The *vertical axis* represents the luciferase activities in cell lysates, expressed as relative light units (RLU) per mg protein. **b** Confocal images of cells transfected with rhodamine-labeled plasmid DNA complexed with R8 or STR-R8 for 3 h. **c** Confocal images of cells transfected with rhodamine-labeled plasmid DNA complexed with FITC-labeled R8 or STR-R8 for 3 h. **d** Atomic force microscopic (AFM) analysis of R8/DNA and STR-R8/DNA complexes. *Bar*, 100 nm

mol% STR-R8, were positively charged and ~100 nm in diameter. CLSM of living cells incubated with R8-Lip containing a rhodamine aqueous phase for 1 h showed that the liposomes were efficiently internalized and located in the cytosol. Surprisingly, internalization of the liposomes was only slightly inhibited at low temperature, indicating that the major uptake pathway is not typical endocytosis (Fig. 7a). Liposomes were internalized as intact vesicles since a high degree of co-localization between the lipid and the aqueous phase markers in the cytosol was found. Furthermore, inhibitors of clathrin-mediated endocytosis slightly inhibited uptake of the liposomes; however, the liposomes were partially co-localized with acidic compartments in the cytosol (Fig. 7b), indicating that a certain fraction of the liposomes are internalized through endocytic pathways that may be different from clathrin-mediated endocytosis. It has

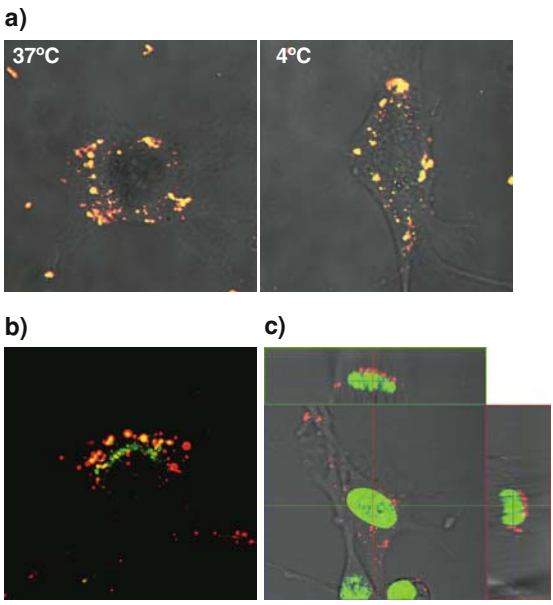

Fig. 7. **a** Images of live cells showing the uptake of R8-Lip containing rhodamine-labeled lipids and FITC-dextran in their aqueous phase at different temperatures. **b** Co-localization between R8-Lip containing rhodamine aqueous phase and FITC-lysosensor after 3 h. **c** Three-dimensional analysis of cells incubated for 9 h with R8-Lip containing a rhodamine aqueous phase. The nucleus was stained with Syto-24 green fluorescence

recently been shown that uptake of the TAT-Cre fusion protein occurs through macropinocytosis (Wadia et al. 2004). Since R8 and TAT peptides would be expected to share a similar internalization pathway (Suzuki et al. 2002), a possible contribution of macropinocytosis in the uptake of R8-Lip is highly likely. It has been suggested that macropinosomes are leaky and do not fuse with lysosomes (Swanson and Watts 1995). Although the exact mechanism is currently unclear, uptake through non-endocytic pathways or through macropinocytosis is still more advantageous than through classical endocytosis, because lysosomal degradation is highly avoided in both cases. Another interesting observation was that, after a 9-h incubation, R8-Lip were located largely in the perinuclear region, bound to the nuclear membrane (Fig. 7c). Therefore, using R8-Lip, the nucleus can be accessed using pathways different from typical endocytosis. This demonstrates the importance of the topology of the peptide in achieving a more preferred entrance pathway, since the interaction between free peptides and plasma membrane seems to be important for the peptide to exert its function.

3.2.4 Transgene Ability of MEND Modified with STR-R8

Based on the mechanistic study of R8-Lip, it was expected that the gene vector MEND modified with STR-R8 would show a high transgene ability. A MEND prototype was constructed using a three-step lipid-film hydration method consisting of: (1) DNA condensation; (2) lipid film hydration; and (3) sonication for lipid coating (Kogure et al. 2004). Packaging of the condensed DNA with the fusiogenic lipid (DOPE/CHEMS) improved transfection activity, possibly due to enhancing the endosomal escape of the complexes (Fig. 8). Surprisingly, as shown in Fig. 8, the localization of STR-R8 on

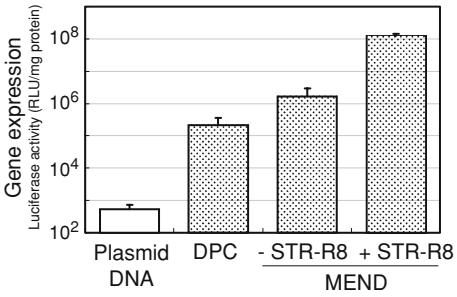

Fig. 8. Transfection assay for plasmid DNA, DNA/polycation complex (DPC), MEND and MEND(+STR-R8) in NIH3T3 cells. Samples containing 0.4 μg DNA suspended in 0.25 ml of serum and antibiotic-free DMEM were added to 4×10^4 NIH3T3 cells and incubated for 3 h at 37°C. One ml of DMEM containing 10% fetal calf serum was added to the cells, followed by a further incubation for 45 h. The cells were then washed, solubilized, and luciferase activity was measured using a luminometer. Luciferase activities are expressed as relative light units (RLU) per mg protein. Data represent the mean±S.D. ($n = 3$)

the surface of the MEND enhanced transfection activity by two orders of magnitude compared to MEND without STR-R8 (Kogure et al. 2004). Furthermore, MEND (+STR-R8) produced transfection activities as high as that of adenovirus, which is one of the most potent viral vectors (unpublished result). A negligible cytotoxicity was observed for the MEND(+STR-R8). Thus, the high transfection ability of MEND(+STR-R8) results from its entrance via a pathway that is different from classical endocytosis.

4 Optimization of Intracellular Trafficking: Pharmacokinetic Considerations

Although transgene expression is governed by various rate-limiting intracellular barriers, most of the current research efforts have focused on individual barriers to enhance transgene expression. However, the increased efficiency of one process may reduce that of others. For example, it is generally accepted that the tight condensation of pDNA, so as to produce a small complex, enhances cellular uptake by endocytosis, but excess condensation inhibits transcription. In order to optimize intracellular trafficking it is necessary to balance all of these processes. A computer-assisted intracellular kinetic model (Fig. 9) with kinetic parameters (i.e. first-order rate constant: $time^{-1}$) determined using quantitative data is a useful tool to analyze, simulate, and optimize transgene expression (Ledley and Ledley 1994; Varga et al. 2001; Banks et al. 2003). Varga et al. (2001) integrated a kinetic model for cellular uptake, endosomal release, nuclear binding, nuclear translocation, dissociation, and protein synthesis with first-order mass action kinetics and demonstrated the utility of kinetic modeling for optimization.

In spite of the great advantages of kinetic modeling, most vector development studies measure final output, e.g., transfection activity, while intracellular events remain a black box, because of the lack of a quantitative assay system for pDNA in

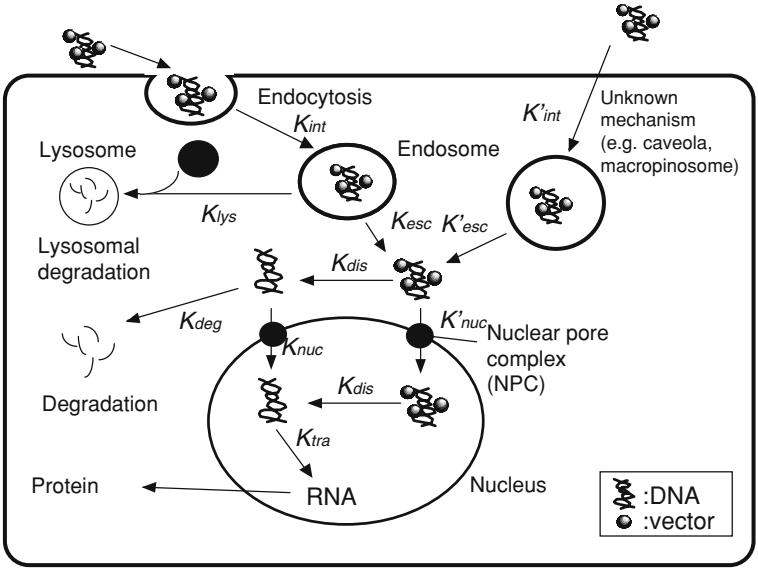

FIG. 9. The intracellular pharmacokinetic model of exogenous DNA. The intracellular disposition of the gene is represented. A complex of DNA and gene vector is internalized via endocytosis (*kint*). Endosomal plasmid DNA fused with lysosome is degraded (*klys*). Alternatively, the complex can enter the cells via a non-classical pathway (*k'int*). A certain fraction of the endosomal DNA is released into the cytosol (*kesc*) where some of the DNA dissociates from the cationic lipids/polycations (*kdis*). Free plasmid DNA is subject to degradation by nucleases (*kdeg*) or partially translocated to the nucleus (*knuc*). Alternatively, DNA enters the nucleus in a complexed form with cationic lipids/polycations (*k'nuc*) followed by dissociation in the nucleus (*kdis*). Finally, free DNA in the nucleus is transcribed (*ktra*)

each organelle. In fact, the kinetic parameters used in the simulation demonstrated by Varga et al. were mainly collected from existing data in the literature, which were evaluated using different cell cultures and different gene vectors.

Recently, we and other researchers established a methodology for quantifying the amount of plasmid DNA in the nucleus by subcellular fractionation of the nucleus followed by the polymerase chain reaction (PCR) for quantification (Leopold et al. 1998; Tachibana et al. 2001; Oh et al. 2002; Tachibana et al. 2002). PCR is a very convenient technique for measuring the amount of pDNA. However, it cannot distinguish intact plasmid DNA from a degraded sample. To resolve this problem, Southern blotting can be applied (Tachibana et al. 2001; Tachibana et al. 2002) to estimate the stability of the plasmid DNA from its length. Using these methods, we were able to clarify the relationship between the applied dose of plasmid DNA, the amount of nuclear DNA, and transgene expression. These data indicated that the nuclear plasmid DNA increased linearly depending on the dose, however, a dramatic saturation of transgene expression was observed. These results indicate that it is necessary to enhance not only the nuclear delivery of plasmid DNA, but also the transcription efficiency of the plasmid itself in the nucleus. In contrast to the nucleus, very few reports are available con-

cerning the amount of plasmid DNA in the endosome/lysosome compartment, and, therefore, it is very difficult to quantitatively evaluate the efficiency of endosomal release. Although the subcellular fractionation of endosomes/lysosomes may solve this problem, several factors, such as the complexity of the steps in the protocol, uncertainties associated with the recovery of the endosomal fraction and mutual contamination of each organelle may preclude the use of this methodology in practical applications.

For these reasons, we have proposed a novel strategy for quantifying the distribution of pDNA in the cytosol, endosomes/lysosomes, and the nucleus simultaneously, using sequential Z-series images captured by CLSM, which is called confocal image-assisted 3-dimensionally integrated quantification (CIDIQ) (Akita et al. 2004). A schematic diagram of this technique and the numerical formulas are provided in Fig. 10. After transfection of rhodamine-labeled pDNA, acidic compartments (endosomes/lysosomes) and the nucleus are stained with LysoSensor DND-189 (green) and Hoechst 33258 (blue), respectively, to determine the subcellular localization of the pDNA. At an early time after transfection, plasmid DNA is detected as clusters. When plasmid DNA is localized in the endosomes/lysosomes, the cytosol, and the nucleus, the molecules are detected as yellow, red, and pink clusters, respectively. For quantification, the pixel areas of the clusters are used as an index of the amount of pDNA. The total pixel area for the clusters of plasmid DNA in each subcellular compartment are first determined in each xy-plane. These values are then further integrated and denoted as S(cyt), S(endosome/lysosome) and S(nucleus), which represent the amount of plasmid DNA in each organelle, in one cell. S(tot), determined by the integration of these values, represents the total cellular uptake of plasmid DNA in one cell. Finally, the fraction of plasmid DNA in each compartment in the total cell is calculated. As shown above, the transgene expression level of STR-R8 was drastically higher than that of R8, while it was much less than that of the commercially available lipoplex, LipofectAMINE PLUS (Fig. 11). In order to clarify which process is rate-determining, this approach was applied to an analysis of the intracellular trafficking of pDNA, transfected by LipofectAMINE PLUS, stearylated octaarginine (STR-R8), and R8. The results showed that most of the pDNA was trapped by endosomes/lysosomes in the case of R8, whereas STR-R8 underwent endosomal escape followed by nuclear translocation in a time-dependent manner. These data suggest that a stearyl moiety enhances the endosomal escape process. Furthermore, LipofectAMINE PLUS was the most effective for rapidly delivering DNA to the nucleus as well as the cytosol. Surprisingly, nuclear localization was observed within 1 h, which is as rapid as that for an adenovirus. This phenomenon is consistent with the fact that transgene expression began to be exhibited within 3 h. Collectively, the differences in transgene expression can be readily explained by intracellular trafficking, as assessed by CIDIQ. In addition, combining the inhibition study of the internalizing process, we succeeded in assessing the contribution of the endocytotic pathway to the total cellular uptake of Lipoplex. Such quantitative data on the contribution of various pathways to overall cellular uptake are essential for establishing intracellular pharmacokinetic models in the future. Collectively, this method can be applied to the intracellular pharmacokinetic analysis of various gene vectors and would be useful in the development of new gene delivery systems. In the future, an application that enables all of these quantification procedures to be automated is needed, since it is necessary to analyze a large number of

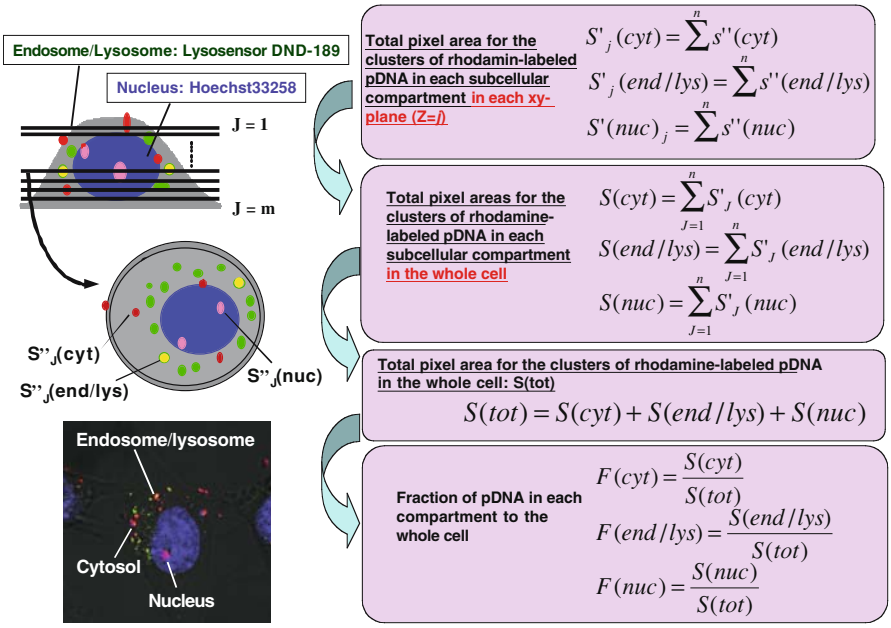

FIG. 10. Methods for quantifying the subcellular distribution of pDNA. After transfection of rhodamine-labeled pDNA, the endosome/lysosome and nuclear fractions were stained with LysoSenser DND-189 and Hoechat33342, respectively, to identify the subcellular localization of pDNA. Each 8-bit TIFF image was transferred to an Image-Pro Plus ver 4.0 (Media Cybernetic, Silver Spring, Md., USA) in order to quantify the total brightness and pixel area of each region of interest. For the data analysis, endosomes/lysosomes, s_i(end/lys), cytosol, s_i (cyt) and nucleus; s_i (nuc), were separately summed up in each xy-plane, and are denoted as $S'_{Z=j}$(mem), $S'_{Z=j}$(end/lys), $S'_{Z=j}$(cyt), and $S'_{Z=j}$(nuc), respectively. The values of $S'_{Z=j}$(mem), $S'_{Z=j}$(end/lys), $S'_{Z=j}$(cyt), and $S'_{Z=j}$(nuc) in each xy-plane were further summed up and are denoted as S(end/lys), S(cyt), and S(nuc), respectively. These parameters represent the total amount of pDNA in each compartment in the whole cell. Furthermore, the total area of the pDNA, denoted as S(tot), was calculated by integrating S(end/lys), S(cyt), and S(nuc). This value represents the total cellular uptake of pDNA. The fractions of pDNA present on, endosomes/lysosomes, cytosol and nucleus relative to the whole cell are denoted as F(end/lys), F(cyt), and F(nuc), which are calculated as S(end/lys), S(cyt), and S(nuc) divided by S(tot), respectively

confocal images in order to obtain an accurate time course for the determination of kinetic parameters by fitting.

5 Conclusions

We have introduced a new concept, Programmed Packaging, for the rational development of a non-viral gene delivery system. Based on this concept, a Multifunctional Envelope-type Nano Device (MEND) was developed that overcomes cellular and intracellular barriers. R8-modified MEND yields transfection activities that are as

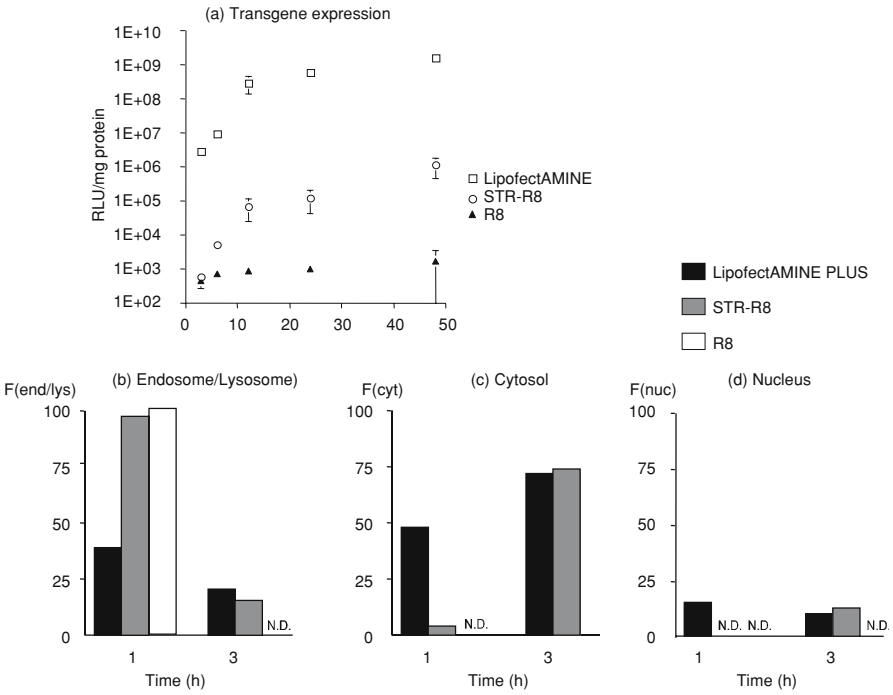

FIG. 11. Transgene expression (**a**) and intracellular disposition (**b–d**) of pDNA transfected by the LipofectAMINE, STR-R8 and R8. **a** pDNA encoding luciferase was condensed with LipofectAMINE PLUS, STR-R8 or R8 and incubated for 3 h at 37°C. One ml of DMEM containing 10% fetal calf serum was added to the cells, followed by a further incubation. At the indicated times, cells were harvested and the luciferase expression level was evaluated. **b–d** At 1 and 3 h post-transfection with LipofectAMINE PLUS, STR-R8 or R8, subcellular distributions of pDNA in the endosome/lysosome (**b**), cytosol (**c**), and nucleus (**d**) were quantified by CIDIC as described in the text

high as those of adenovirus under in vitro conditions. A quantitative assay of the intracellular trafficking of the gene delivery system (CIDIQ) will clarify the rate-limiting step of non-viral vectors, which will be useful for the development of nano-machines.

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