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Note

Enhancement of transfection activity of lipoplexes by complexation with transferrin-bearing fusogenic polymer-modified liposomes

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Abstract

We previously developed complexes of lipoplexes containing 3β-(*N*-(*N*′,*N*′-dimethylaminoethane)carbamoyl)cholesterol (DC-chol) and succinylated poly(glycidol)-modified liposome, which becomes fusogenic under weakly acidic condition, for use as a novel gene delivery system. This study explored the effect of lipoplex structures – the type of cationic lipid and cationic lipid/DNA charge ratio – on the transfection activity of those complexes. Three types of cationic lipid with different polar groups were used for the preparation of lipoplexes: DC-chol, *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate (DOTAP), and 3,5-dipentadecyloxybenzamidine (TRX-20) with dimethylamino group, trimethylammonium group, and benzamidine group, respectively. Complexation with the SucPG-modified transferrin-bearing liposomes affected transfection activity of these lipoplexes differently. The TRX-20 lipoplexes exhibited the most marked enhancement of transfection activity upon complexation with the SucPG-modified liposomes among these lipoplexes. The cationic lipid/DNA charge ratio of the lipoplex and the amount of the transferrin-bearing SucPG-modified liposomes associated to the lipoplex also affected the transfection activity of the resultant complexes. Highly potent gene vectors were obtained by adjusting these factors.

Keywords: Gene delivery; Fusogenic liposome; Cationic liposome; Transferrin; Lipoplex

Numerous efforts have been made to develop efficient non-viral vectors for gene therapy (Brown et al., 2001; Niidome and Huang, 2002). Lipoplexes and polyplexes are respective complexes of cationic liposomes and cationic polymers with DNA. They are currently viewed as promising systems, but their activity can be improved. These systems bind to the cell surface through electrostatic interactions and are taken up by cells mainly via endocytosis. Subsequently, some parts of the gene contained in the complexes reach the nucleus, where gene transcription occurs. However, most of the complexes are likely to be trapped in the endosome, to be degraded eventually in the lysosome (Friend et al., 1996, Mönkkönen and Urtti, 1998). Therefore, to achieve efficient transfection of cells, gene vectors must possess an ability to promote gene transferral from the endosome to the cytosol before degradation in the lysosome (Zabner et al., 1995; Pouton and Seymour, 1998). Various methods have been used to enhance the endosomal escape of the entrapped gene, such as the use of membrane active molecules (Wagner, 1999, Kamata et al., 1994, Simoes et al., 1998, Kichler et al., 1997) and proton sponge effect (Sonawane et al., 2003; Boussif et al., 1995; Takahashi et al., 2003, 2005). Especially for lipoplexes, induction of fusion with endosome is one of the most efficient approaches to promote transfer of gene into cytosol and increase transfection efficiency (Farhood et al., 1995; Mok and Cullis, 1997).

In a previous study (Kono et al., 2001), we prepared complexes of lipoplexes and liposomes modified with transferrin-bearing succinylated poly(glycidol) (SucPG), which generate fusogenic activity at mildly acidic pH (Fig. 1) (Kono et al., 1994, 1997). These complexes, which are termed SucPG complexes, were designed to achieve cell transfection through efficient internalization into cells through transferrin receptor-mediated

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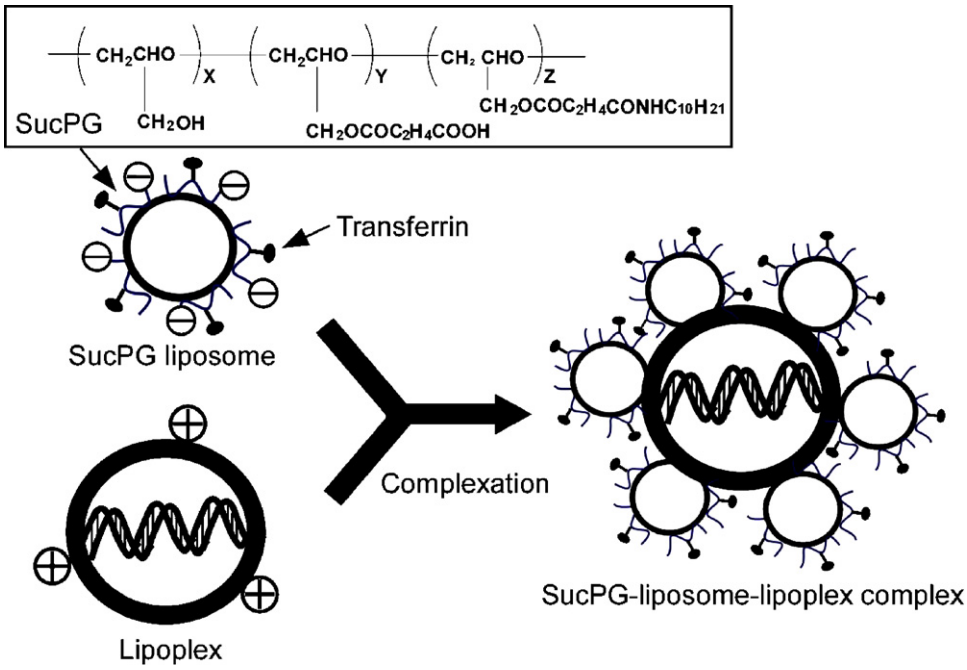

Fig. 1. Structure of SucPG having *n*-decyl groups and preparation of SucPG-complex bearing transferrin.

endocytosis and subsequent release of DNA into the cytoplasm by fusion with endosomal membrane. These complexes have a structure in which negatively charged SucPG-modified liposomes are associated with positively charged lipoplexes through electrostatic interaction. Transfection activity of DC-chol lipoplexes was actually enhanced by complexation with SucPG-modified liposomes. However, it remains unknown how the structure and properties of lipoplexes affect their transfection activity upon complexation with the SucPG-modified transferrin-bearing liposomes. To elucidate those questions, this study prepared lipoplexes from cationic lipids having different types of head groups: DC-chol with dimethylamino group, DOTAP with trimethylammonium group, and TRX-20 with benzamidine group (Fig. 2) (Harigai et al., 2001). Using them, we examined the effect of complexation of these lipoplexes with the transferrin-bearing SucPG liposomes on their transfection activity. Here, we report the influence of lipoplexes' structural properties – the type of cationic lipids and charges – on the fusogenic liposome-mediated enhancement of transfection activity of the lipoplexes.

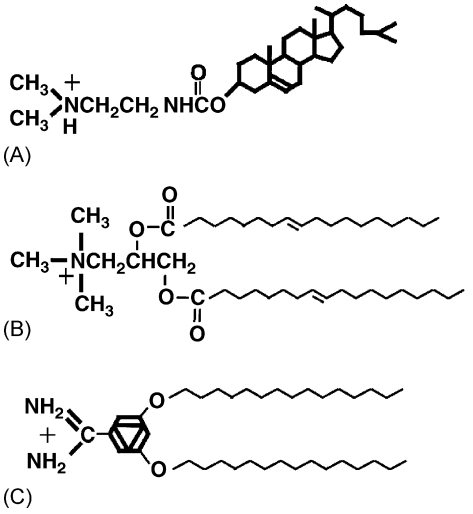

Fig. 2. Structures of DC-chol (A), DOTAP (B) and TRX-20 (C).

For the preparation of lipoplexes, cationic liposomes with three kinds of cationic lipids: DC-chol, DOTAP, and TRX-20 were used. These cationic liposomes contained phospholipids, such as DOPE and DLPC, in addition to cationic lipids. Compositions of these cationic liposomes were optimized to exhibit high transfection activity and determined as follows: DC-chol/DOPE (45:55, mol/mol); DOTAP/DOPE (1:1, mol/mol); TRX-20/DOPE/DLPC (1:2:1, mol/mol/mol).

Lipoplexes and SucPG complexes were prepared as reported previously (Kono et al., 2001). SucPG with the composition (x:y:z, Fig. 1) of 18:74:8 (mol/mol/mol) and the number-average molecular weight of 23,500 was used. These cationic liposomes were incubated with plasmid p10[18,23], which contains a firefly luciferase coding sequence derived from pGL3-basic (Promega) between Raus sarcoma virus LTR and polyadenylation signal in phosphate-buffered saline (PBS) for 10 min. Also, SucPG-modified liposomes were prepared by suspending a mixture of SucPG and egg yolk phosphatidylcholine in PBS and subsequent extrusion through a polycarbonate membrane with a pore size of 50 nm and conjugation of transferrin using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide as previously reported (Kono et al., 2001).

Table 1 lists the zeta potential and diameter of liposomes used in this study. The diameter of SucPG-modified liposomes was 65 nm, which was approximately the pore size of the polycarbonate membrane used for liposome extrusion. The zeta potential of the SucPG-modified liposomes indicates that these liposomes

Table 1
Diameter and zeta potential of liposomes

Liposome	Diameter (nm)	Zeta potential (mV)
TRX-20	146 ± 29	35.6 ± 3.5
DC-chol	201 ± 40	27.3 ± 2.3
DOTAP	141 ± 35	27.1 ± 2.4
SucPG	65 ± 13	−25.1 ± 2.9

Measurements were performed at 25 °C at pH 7.

possess negatively charged surfaces because of their negatively charged carboxylate groups on the SucPG chains. On the other hand, diameters and zeta potentials of these cationic liposomes were, respectively, between 140 and 200 nm and between 27 and 36 mV. Thus, irrespective of cationic lipid types, these liposomes possess similar sizes and surface charge densities.

The SucPG complexes were prepared by incubating the lipoplexes with varying amounts of the SucPG-modified liposome bearing transferrin for 5 min in an ice bath according to the method previously reported (Kono et al., 2001). Fig. 3A represents zeta potentials of various lipoplexes and their complexes with varying amounts of SucPG-modified liposomes. These lipoplexes exhibited zeta potentials around 20–27 mV. However, their zeta potentials decreased with increasing amount of SucPG-modified liposomes added to the lipoplexes, indicating that these lipoplexes formed complexes with SucPG-modified liposomes with similar efficiency. Their zeta potentials reached a constant value of about −20 mV at the succinylated unit/nucleotide unit ratio above 10, implying that the lipoplexes were covered with the SucPG-modified liposomes.

Diameters of the complexes of the lipoplexes–SucPG-modified liposomes were evaluated using dynamic light scattering (Fig. 3B). Their diameters changed depending on their composition and exhibited maximum at the succinylated unit/nucleotide unit ratio around four to seven, where the complexes became electrically neutral (Fig. 3A). Complex formation between the lipoplex and SucPG-modified liposomes was further confirmed from their morphology using atomic force microscopy. The TRX-20 lipoplex had a smooth surface, but a highly rough surface with many projections of about

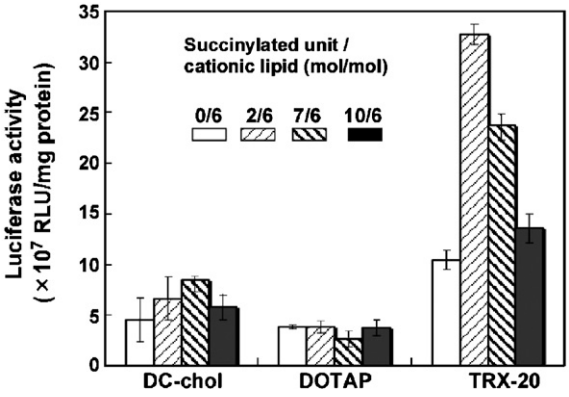

Fig. 4. Transfection activity of SucPG complexes prepared by incubating DC-chol, DOTAP and TRX-20 lipoplexes with varying amounts of SucPG-modified liposomes bearing transferrin. Luciferase activities of HeLa cells treated with SucPG complexes and their parent lipoplexes (open bars) are shown. The lipid/DNA (+/−) charge ratio of the lipoplexes was 6. The compositions of SucPG complexes are expressed as succinylated unit/cationic lipid (mol/mol) ratio in the figure. Each bar is the mean ± S.D. ($n = 3$). The cells (5×10^4) were treated with vectors containing 1 μg DNA in the presence of 10% FCS.

70–100 nm was observed after the complexation with SucPG-modified liposomes, suggesting that SucPG-modified liposomes were adsorbed onto the lipoplexes (results not shown).

The effect of complexation with SucPG-modified liposomes on transfection activity of these lipoplexes was examined. Fig. 4 presents expression of luciferase gene in HeLa cells treated with various complexes prepared by mixing, at varying ratios, lipoplexes of various types and the SucPG-modified transferrin-bearing liposomes. That figure shows that the effect of the fusogenic liposome complexation varies depending on the lipoplex type, even though these lipoplexes have equal cationic lipid/DNA phosphate (mol/mol) ratios. The transfection activity of the DOTAP lipoplex hardly changed, irrespective of the amount of the SucPG-modified liposomes mixed. In contrast, the TRX-20 lipoplex activity was markedly enhanced by addition of the fusogenic liposomes. The produced SucPG-complex exhibited a remarkably higher transfection activity than the parent TRX lipoplex when an appropriate amount of SucPG-modified

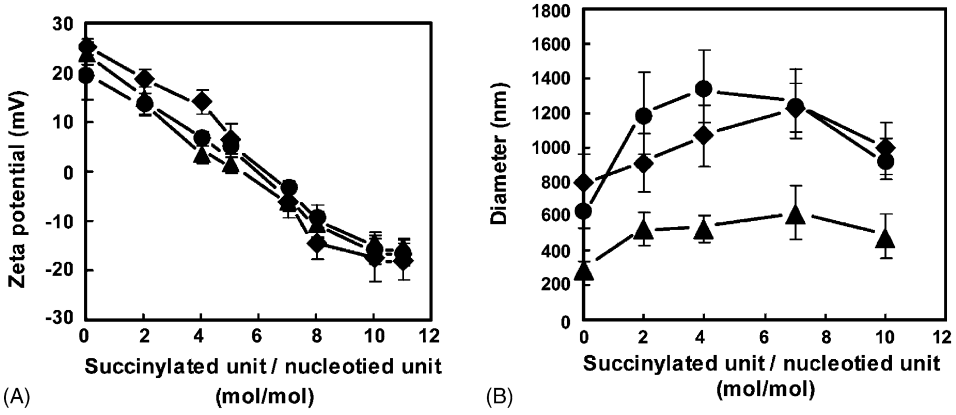

Fig. 3. Zeta potentials (A) and diameters (B) of SucPG-complexes prepared by incubating DC-chol (circles), DOTAP (triangles) and TRX-20 (diamonds) lipoplexes with varying amounts of SucPG-modified liposomes. The lipid/DNA (+/−) charge ratio of the lipoplexes was 6. The compositions of SucPG complexes are expressed as succinylated unit/DNA nucleotide unit ratio in the figure.

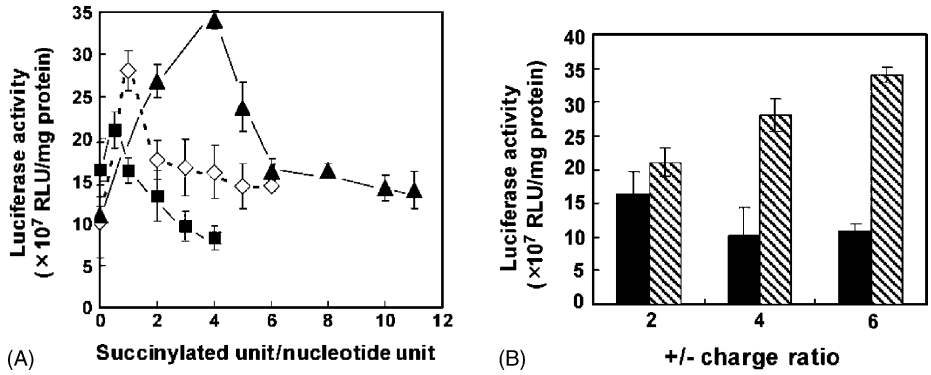

Fig. 5. Transfection activity of SucPG complexes prepared by complexation of TRX-20 lipoplexes and varying amounts of transferrin-bearing SucPG-modified liposomes. TRX-20 lipoplexes with the lipid/DNA (+/−) charge ratios of 2 (squares), 4 (diamonds), and 6 (triangles) were used as the starting lipoplexes. (A) Effect of composition on transfection activity of the complexes. The composition of the complex was expressed as the molar ratio of succinylated unit of SucPG-modified liposomes to DNA nucleotide units of the lipoplex. (B) Comparison of transfection activities between the SucPG complexes with optimum composition (hatched bars) and their parent lipoplexes (closed bars) with the lipid/DNA (+/−) charge ratios of 2, 4, and 6. Each point (A) or bar (B) is the mean ± S.D. ($n = 3$). The cells (5×10^4) were treated with vectors containing 1 μg DNA in the presence of 10% FCS.

liposomes was mixed to these lipoplexes. A similar tendency was shown for the DC-chol lipoplex. However, transfection activity of the DC-chol lipoplex was lower than that of the TRX-20 lipoplex and promotion of activity by complexation with the SucPG-modified liposomes was less marked than in the case of TRX-20 lipoplex.

Next we examined the influence of the cationic lipid/DNA (+/−) charge ratio of the TRX-20 lipoplexes on activity of the resultant SucPG-complexes because the TRX-20 lipoplex showed remarkable enhancement of transfection activity by complexation with SucPG-modified liposomes. The TRX-20 lipoplexes with the +/− charge ratios of 2, 4 and 6 were used for complexation with SucPG-modified liposomes. Fig. 5A shows that, irrespective of charge ratios, the transfection activity of the lipoplexes rose with increasing amount of the SucPG-modified liposomes added. However, complexation using too much SucPG-modified liposome degraded the activity. Fusion ability of the complexes increases concomitant with an increasing amount of the associated SucPG-modified liposomes, whereas the addition of too much SucPG-modified liposome might cause appearance of unassociated SucPG-modified liposome. These free SucPG-modified liposomes bear transferrin on the surface. Therefore, they might inhibit binding of the liposome–lipoplex complex to the cells because of interaction between the transferrin and its receptor. Consequently, appropriate amounts of the SucPG-modified liposomes were mixed with the lipoplexes to obtain maximum activity. The lipoplexes with charge ratios of 2, 4, and 6, respectively, produced SucPG complexes with maximum activity at the succinylated unit/phosphate unit ratios of 0.5, 1.0 and 4.0. Though the lipoplex with a high +/− charge ratio requires more SucPG-modified liposomes to achieve maximum activity, stronger activity is obtainable using lipoplexes with a higher +/− charge ratio.

Fig. 5B depicts transfection activities of the SucPG-complexes with the optimum composition and their parent lipoplexes. The SucPG complexes' activity increased remarkably with increasing +/− charge ratio of the lipoplexes used for their preparation. However, the activity of the parent lipoplexes tends to decrease concomitant with an increased charge ratio. This result indicates that activity of the SucPG complexes does not correlate with the activity of the parent lipoplex. The SucPG-complex made from the lipoplex with a high +/− charge ratio contained more SucPG-modified liposomes in the optimized composition. This complex might therefore have higher fusion ability and stronger affinity to the cell surface because of the transferrin–transferrin receptor interaction, implying stronger transfection activity.

This study has demonstrated that complexation of lipoplexes with the transferrin-bearing SucPG-modified liposomes is an effective method to obtain potent non-viral vectors that achieve gene transfection of cells through specific interaction between transferrin and its receptor. The effect of their complexation on transfection activity of the resultant complex depends on structural properties of lipoplex, such as the type of cationic lipid and the lipid/DNA charge ratio, and the amount of the SucPG-modified liposomes associated to the lipoplex. Because some complexes obtained by using TRX-20-based lipoplexes with appropriate charge ratios and the amount of the associated SucPG-modified liposomes achieved more efficient transfection of HeLa cells than widely used transfection reagents, such as DC-chol and DOTAP, these complexes are considered to be a promising candidate of non-viral vectors. In addition, complexation of the ligand-bearing SucPG-modified liposomes with other types of vectors may generate novel types of potent non-viral vectors.

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