Properties of Cationic Liposomes Composed of Cationic Lipid YKS-220 Having an Ester Linkage: Adequate Stability, High Transfection Efficiency, and Low Cytotoxicity

Satoshi OBIKA, Wei YU, Atsuko SHIMOYAMA, Takeshi UNEDA, Takashi MINAMI, Kazuyuki MIYASHITA, Takefumi DOI, and Takeshi IMANISHI*

Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan.

Received August 24, 1998; accepted November 10, 1998

Cationic lipid *N*-[3-[2-(1,3-dioleoyloxy)propoxy-carbonyl]propyl]-*N,N,N*-trimethylammonium iodide (YKS-220) having a symmetrical and biodegradable structure was employed for the preparation of cationic liposomes with dioleoylphosphatidylethanolamine (DOPE). The stability, transfection activity in several cell lines and cytotoxicity of YKS-220 cationic liposomes were studied. It was found the YKS-220 cationic liposomes were very stable and their transfection activity remained even after storage at 4 °C for 12 months. The transfection activity of these liposomes was assayed using CHO, COS, and HepG2 cells and found to be comparable with, or better than, that of other cationic liposomes, such as *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methylsulfate (DOTAP) liposome, *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium chloride (DOTMA) liposome (Lipofectin®), and 2,3-dioleoyloxy-*N*-[2-(sperminecarboxamido)ethyl]-*N,N*-dimethyl-1-propanaminium trifluoroacetate (DOSPA) liposome (LipofectAMINE™). In addition, the cytotoxicity of YKS-220 cationic liposomes was far lower than that of other cationic liposomes.

Key words cationic liposome; cationic lipid; gene transfer; ester linkage

Since cationic lipid *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium chloride (DOTMA) was first employed for gene transfer into living cells in 1987,¹⁾ many novel cationic lipids have been designed, synthesized and used for DNA transfection *in vitro*^{1–9)} or *in vivo*.^{7,8)} To reduce the cytotoxicity of DOTMA, various forms of linkage, such as ester, amide and carbamoyl groups, were introduced between the hydrophilic head group and the hydrophobic tail group of cationic lipids,^{4–6)} instead of the biologically and chemically stable ether linkage. Among these linkages, the ester linkage was considered to be unstable during the preparation or storage of cationic liposomes.^{5,9)} Although cationic lipids having an ester linkage were demonstrated to be less toxic than those having an ether linkage,^{5,10,11)} almost all the novel lipids reported so far have an ether, amide or carbamoyl linkage which is relatively stable under biological conditions. We have tried to answer the question: is a cationic lipid containing an ester linkage really so unfavorable for use in DNA transfection?

Nowadays, almost all cationic liposomes contain dioleoylphosphatidylethanolamine (DOPE) as a helper lipid, which also reduces the cytotoxicity.¹²⁾ It is well known that DOPE can rupture or destabilize the endosomal membrane to help DNA escape hydrolysis in lysosomes because of their high non-bilayer forming activity.^{3,13–15)} The bilayer form of DOPE can be stabilized by various agents such as proteins or lipids.^{16–18)}

In a previous paper, we reported the synthesis of a novel cationic lipid *N*-[3-[2-(1,3-dioleoyloxy)propoxycarbonyl]propyl]-*N,N,N*-trimethylammonium iodide (YKS-220), which has a biodegradable ester linkage and a symmetrical structure. We also showed that the cationic liposome composed of YKS-220 and DOPE (YKS-220 : DOPE = 1 : 4, mol ratio) exhibited high transfection activity in CHO cells and lower cytotoxicity than DOTMA (Lipofectin®) and 2,3-dioleoyloxy-*N*-[2-(sperminecarboxamido)ethyl]-*N,N*-dimethyl-1-propana-

minium trifluoroacetate (DOSPA) (LipofectAMINE™) liposomes, both of which have a stable ether linkage.¹¹⁾ Here, we report the stability and transfection efficiency in several cell lines as well as the cytotoxicity of YKS-220 cationic liposomes compared with *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methylsulfate (DOTAP) having an ester linkage.

MATERIALS AND METHODS

Materials The YKS-220 cationic lipid was prepared according to the procedure reported previously.¹¹⁾ The structures of YKS-220 and other cationic lipids are shown in Fig. 1. DOPE and a luciferase expression plasmid pGVC were purchased from Wako Pure Chemical Industries (Osaka, Japan). Lipofectin®, LipofectAMINE™ and OPTI-MEM® were purchased from Gibco BRL (NY, U.S.A.). DOTAP was obtained from Boehringer Mannheim GmbH (Mannheim, Germany). A luciferase expression vector pRSVL¹⁹⁾ was kindly provided by Dr. M. Nakanishi (Research Institute for Microbial Disease, Osaka University).

Fig. 1. The Structures of YKS-220 and Other Representative Cationic Lipids

* To whom correspondence should be addressed.

Preparation of YKS-220 Cationic Liposomes YKS-220 cationic liposomes were prepared as follows. A chloroform solution (0.5 ml) of YKS-220 (0.2 μmol) and DOPE (0.8 μmol) in a vial was concentrated by rotary evaporation to form a lipid film on the wall. The vial was placed under vacuum for 4 h to remove the remaining solvent, then 0.5 ml deionized water was added and vortexed for 2 min at room temperature to produce multilamellar vesicles (MLV). Further sonication with a probe-type sonicator W-220F (Heat-systems-Ultrasonics Inc., NY, U.S.A.) for 5 min under nitrogen gave small unilamellar vesicles (SUV). The SUV liposomes were filtered through a 0.2 μm filter to sterilize them. The size of the liposomes was determined using a submicron laser light scattering instrument LPA-3000/3100 (Otsuka Electronics Co., Ltd., Osaka, Japan).

Formation of the Liposome-DNA Complex The luciferase expression plasmid (pRSVL or pGVC) and YKS-220 cationic liposomes, both of which had been separately diluted with 1 ml OPTI-MEM[®] reduced-serum medium, were mixed and then allowed to stand at room temperature for 15 min. For a typical transfection, 1 μg plasmid DNA complexed with 20 nmol liposome per 35-mm plate used.

Cationic Liposome-Mediated Transfection into Cells The cells were inoculated at a density of 1×10^5 cells per 35-mm plate, incubated at 37°C for 18 h in 2 ml Ham's F-12 medium for CHO cells, Dulbecco's modified Eagle medium (DMEM) for COS and HepG2 cells supplemented with 10% fetal bovine serum (FBS) and 2% antibiotic in a 5% CO₂ atmosphere. To the cell culture plates, which had been washed twice with phosphate-buffered saline (PBS), 1.9 ml OPTI-MEM[®] medium without FBS and 0.1 ml liposome-DNA complex (typical condition: liposome 10 μM ; DNA 0.5 $\mu\text{g}/\text{ml}$) were added. After incubation for 24 h at 37°C, luciferase activity was measured as described below.

Luciferase Assay Luciferase activity was measured using a luciferase assay (PicaGene[™], Toyo Inki Co., Ltd., Tokyo, Japan) in a luminometer (Lumat LB9501, EG and G Berthold, Bad Wildbad, Germany). The activity was reported as relative light units (RLU) per μg protein.

Cytotoxicity Assay The effect of the cationic liposome on cell growth was assessed by a colorimetric dye reduction assay (Cell Counting Kit, Wako Pure Chemical Industries, Ltd., Osaka, Japan). CHO cells were seeded at a density of 1×10^3 cells per well, and incubated at 37°C for 18 h in 100 μl Ham's F-12 medium supplemented with 10% FBS and 2% antibiotic in a 5% CO₂ atmosphere. To the cell cultures, 100 μl Ham's F-12 medium containing 0–320 μM cationic liposomes (final concentration 0–160 μM) was added. After incubation for 24 h at 37°C, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-1, 3.3 mg/ml) was added, 10 μl per well, and the plate was incubated at 37°C for 4 h, then at 4°C for 10 min. Absorbance at 450 nm was measured using a plate reader (Model 450, Bio-Rad, CA, U.S.A.).

RESULTS

Stability of YKS-220 Cationic Liposomes In a previous paper, we reported that a 1:4 mol ratio of YKS-220 to DOPE is optimal for the YKS-220 cationic liposome-mediated DNA transfection in the CHO cell line.¹¹⁾ Here, we in-

Fig. 2. Time-Course of Transfection Activity ($\square$) and Mean Diameter ($\blacklozenge$) of YKS-220 Cationic Liposome Stored at 4°C

YKS-220 cationic liposomes were prepared and stored at 4°C for the indicated periods. CHO cells were incubated with the YKS-220 cationic liposome-pGVC complex (YKS-220 cationic liposome 10 μM ; DNA 0.5 $\mu\text{g}/\text{ml}$) at 37°C for 24 h. The cells were harvested and luciferase activity was determined as described in Materials and Methods. Transfection efficiency is expressed as mean $\pm$ S.D. ($n=3$).

Fig. 3. Luciferase Activity Levels in CHO Cells Transfected with the YKS-220 Cationic Liposome-pGVC Complexes Expressed as a Function of DNA Concentration

CHO cells were incubated with the YKS-220 cationic liposome-pGVC complex at 37°C for 24 h. The cells were harvested and luciferase activity was determined as described in Materials and Methods. The final concentration of the liposomes was 2.5 μM ($\square$), 5 μM ($\square$), 10 μM ($\square$), 20 μM ($\square$). The value above the bar shows the charge ratio (+/-) of the liposome-DNA complex. Each point represents a mean value ($n=3$).

vestigated the stability of these liposomes. YKS-220 cationic liposomes were prepared (as described in Materials and Methods) and stored at 4°C. To determine the stability of the YKS-220 cationic liposomes, their size and transfection efficiency were assayed 0, 2, 6 and 12 months after preparation. As shown in Fig. 2, no obvious change in diameter or transfection activity was detected for up to 12 months.

The Optimal DNA Ratio for Cationic Liposome Transfection in the CHO Cell Line It is well known that the ratio of cationic lipid to DNA is important for liposome-mediated transfection.²⁰⁾ In general, cationic liposome-DNA complexes bearing a small net plus charge are thought to be easily adsorbed to the negatively charged cell surface and have a high transfection activity. In order to determine the optimal ratio of the cationic liposomes and DNA, the YKS-220 cationic liposomes were added to plasmid DNA at various liposome and DNA concentrations. The positive to negative charge ratio for these liposome-DNA complexes ranged from 0.3 to 35. The results presented in Fig. 3 show that the

Fig. 4. Transfection Efficiency of YKS-220 Cationic Liposomes Compared with Other Cationic Liposomes, DOTAP, Lipofectin® and LipofectAMINE™ in CHO Cells (A), in COS Cells (B) and in HepG2 Cells (C)

CHO cells were incubated with the liposome-pGVC (0.5 μg/ml) complexes, and COS cells and HepG2 cells were incubated with the liposome-pRSVL (0.5 μg/ml) complexes at 37 °C for 24 h. The amount of YKS-220 cationic liposomes, DOTAP, Lipofectin® and LipofectAMINE™ was 10, 10, 4 and 5 μM, respectively. The cells were harvested and luciferase activity was determined as described in Materials and Methods. The value above the bar shows the relative luciferase activity. Data are expressed as means ± S.D. (n = 3).

optimal charge ratio (+/−) for these complexes is 1.1 and also, at this optimal charge ratio, the luciferase activity is linearly related to the amount of liposome–DNA complex added.

Transfection Efficiency of YKS-220 Cationic Liposomes in Several Cell Lines To evaluate the efficiency of YKS-220 cationic liposome-mediated DNA transfection, the plasmid pGVC or pRSVL encoding the luciferase gene was transfected into several cell lines with YKS-220 cationic liposomes and other commercially available cationic liposomes, such as DOTAP, Lipofectin® (DOTMA:DOPE = 1.2:1, mol ratio) and LipofectAMINE™ (DOSPA:DOPE = 1.5:1, mol ratio) for comparison (Fig. 4). The results show that YKS-220 cationic liposomes have a high transfection efficiency compared with the other cationic liposomes in CHO, COS and HepG2 cell lines.

Cytotoxicity of YKS-220 Cationic Liposomes In order to compare the cytotoxicity of YKS-220 cationic liposomes with that of DOTAP liposomes, we examined the effects of these liposomes on the viability of CHO cells by means of the WST-1 method (modified 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) method). The cytotoxicity of Lipofectin® and LipofectAMINE™ was used as a

Table 1. Cytotoxicity of YKS-220 and Other Cationic Liposomes on CHO Cells

Concentration (μM)	Cell viability (%)			
	YKS-220	DOTAP	Lipofectin	LipofectAMINE
10	91 ± 7	100 ± 6	77 ± 13	100 ± 2
20	97 ± 4	102 ± 4	59 ± 11	119 ± 5
40	106 ± 3	115 ± 7	30 ± 8	109 ± 8
80	115 ± 11	95 ± 14	19 ± 4	22 ± 3
160	111 ± 9	26 ± 5	19 ± 5	23 ± 4

Cell viability of different cationic liposomes at various concentrations was measured by WST-1 method and expressed as a percentage of the control. Data represent the mean ± S.D. (n = 3).

comparison. As shown in Table 1, YKS-220 cationic liposomes were devoid of any cytotoxicity at up to 160 μM total lipids. However, the other three liposomes were cytotoxic at this concentration. In particular, Lipofectin® and LipofectAMINE™ were markedly cytotoxic at 40 and 80 μM, respectively. DOTAP, containing an ester linkage, was found to be less cytotoxic than Lipofectin® and LipofectAMINE™. However, YKS-220 cationic liposomes were less cytotoxic than DOTAP liposomes. Usually, YKS-220 cationic liposomes were used at a concentration of 10 μM for DNA transfection. These liposomes, therefore, do not exhibit any cytotoxicity in routine use.

DISCUSSION

If a biochemically stable, unnatural lipid remained in cells for a long time, it would damage them.¹⁵⁾ The cationic lipid YKS-220, having an ester linkage, has advantages over other cationic lipids due to the fact that it is biodegraded inside cells. However, some researchers have observed that the ester linkage could be cleaved during liposome preparation or storage.^{5,7)} We, therefore, investigated the stability of YKS-220 cationic liposomes during preparation and storage. We found that there was no obvious change in transfection activity and liposome size over one year. Considering that the ester structure is stable under neutral conditions, our results are not unexpected.

YKS-220 cationic liposomes exhibited high transfection activity. These results could be attributed to the high DOPE content of the YKS-220 cationic liposomes (80% DOPE) and the excellent bilayer-stabilizing effect of YKS-220. It is widely accepted that DNA–cationic liposome complexes enter cells by endocytosis.^{13,21,22)} In the acidified endosomes, the helper lipid DOPE is thought to form an inverted non-bilayer which destabilizes the endosomal membrane, helping DNA enter the cytoplasm. The transfection activity of cationic liposomes with a high ratio of DOPE depends on the stabilizing effect of the cationic lipid. Most cationic liposomes previously reported contain about 50% DOPE. Cationic liposomes with a relatively high DOPE content were thought to be unstable and easily transformed into an H_{II} hexagonal structure.²²⁾ This non-bilayer form, produced during the preparation of the liposomes, not only affects the storage period but also causes unfavorable lipid aggregation during the formation of DNA–liposome complexes.²³⁾ We did not observe any obvious change in the size of the YKS-220 cationic liposome, and also found that the transfection activ-

ity in several cell lines is high, suggesting that YKS-220 can inhibit the formation of such a non-bilayer during storage and mixing.

The cytotoxicity of YKS-220 cationic liposomes was, as we expected, far lower than that of DOTMA (Lipofectin®) and DOSPA (LipofectAMINE™) liposomes. Furthermore, YKS-220 cationic liposomes were found to be less cytotoxic than DOTAP liposome which also have an ester linkage. These findings suggest that the low cytotoxicity of YKS-220 cationic liposome is due to both the biodegradable chemical structure of YKS-220 and the high DOPE content of YKS-220 liposomes.

In summary, YKS-220 containing an ester linkage is quite stable for one year and is also an excellent bilayer stabilizer. It is apparent from these studies that YKS-220 cationic liposomes are less cytotoxic and act as a more efficient DNA transfection vector.

Acknowledgment Part of this work was supported by a Grant-in-Aid for Scientific Research, No. 09557201, from the Ministry of Education, Science, Sports and Culture, Japan.

REFERENCES

- 1) Felgner P. L., Gadek T. R., Holm M., Roman R., Chan H. W., Wenz M., Northrop J. P., Ringold G. M., Danielsen M., *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 7413—7417 (1987).
- 2) Felgner P. L., Ringold G. M., *Nature* (London), **337**, 387—388 (1989).
- 3) Felgner J. H., Kumar R., Sridhar C. N., Wheeler C. J., Tsai Y. J., Border R., Ramsey P., Martin M., Felgner P. L., *J. Biol. Chem.*, **269**, 2550—2561 (1994).
- 4) Leventis R., Silvius J. R., *Biochim. Biophys. Acta*, **1023**, 124—132 (1990).
- 5) Gao X., Huang L., *Biochem. Biophys. Res. Commun.*, **179**, 280—285 (1991).
- 6) Behr J. P., Demeneix B., Loeffler J. P., Perez-Mutul J., *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 6982—6986 (1989).
- 7) Nabel G. J., Nabel E. G., Yang Z. Y., Fox B. A., Plautz G. E., Gao X., Huang L., Shu S., Gordon D., Chang A. E., *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 11307—11311 (1993).
- 8) Parker S. E., Ducharme S., Norman J., Wheeler C. J., *Hum. Gene Ther.*, **8**, 393—401 (1997).
- 9) Farhood H., Bottega R., Epand R. M., Huang L., *Biochim. Biophys. Acta*, **1111**, 239—246 (1992).
- 10) Yagi K., Noda H., Kurono M., Ohishi N., *Biochem. Biophys. Res. Commun.*, **196**, 1042—1048 (1993).
- 11) Obika S., Yu W., Shimoyama A., Uneda T., Miyashita K., Doi T., Imanishi T., *Bioorg. Med. Chem. Lett.*, **7**, 1817—1820 (1997).
- 12) Pinnaduwaige P., Schmitt L., Huang L., *Biochim. Biophys. Acta*, **985**, 33—37 (1989).
- 13) Zhou X., Huang L., *Biochim. Biophys. Acta*, **1189**, 195—203 (1994).
- 14) Farhood H., Serbina N., Huang L., *Biochim. Biophys. Acta*, **1235**, 289—295 (1995).
- 15) Burger H. J., Schuetz J. D., Schuetz E. G., Guzelian P. S., *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 2145—2149 (1992).
- 16) Cullis P. R., De Kruijff B., *Biochim. Biophys. Acta*, **559**, 399—420 (1979).
- 17) Litzinger D. C., Huang L., *Biochim. Biophys. Acta*, **1113**, 201—227 (1992).
- 18) Tari A., Huang L., *Biochemistry*, **28**, 7708—7712 (1989).
- 19) De Wet J. R., Wood K. V., DeLuca M., Helinski D. R., Subramani S., *Mol. Cell. Biol.*, **7**, 725—735 (1987).
- 20) Eastman S. J., Siegel C., Tousignant J., Smith A. E., Cheng S. H., Scheule R. K., *Biochim. Biophys. Acta*, **1325**, 41—62 (1997).
- 21) Legendre J. Y., Szoka F. C., Jr., *Pharm. Res.*, **9**, 1235—1242 (1992).
- 22) Zabner J., Fasbender A. J., Moninger T., Poellinger K. A., Welsh M. J., *J. Biol. Chem.*, **270**, 18997—19007 (1995).
- 23) Sternberg B., *J. Liposome Res.*, **6**, 515—533 (1996).