Efficient Nonviral Gene Transfer Mediated by Polyethylenimine in an Insect Cell Line

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(Received November 1, 2004; Accepted November 18, 2004)

An enhanced gene delivery system, monitored by the expression of a luciferase gene, was devised for an insect cell line BmN4, by altering the ratios of several liposome components. First, three kinds of commercially available polyethylenimine (PEI) reagents with different sizes were tested; one (PEI reagent with an average molecular weight of 1,800) gave a much better transfection efficiency than others. Using this particular reagent (PEI-1800), we newly formulated a liposome composed of three polycationic lipids: dioleoylphosphatidylcholine (DOTAP), dioleoylphosphatidylethanolamine (DOPE) and PEI-1800 in a ratio of 1:1:1. The current liposome gave a 2.5-fold efficiency compared to a commercially available liposome (CellFECTIN), a 2:3 mixture of TM-TPS (tetramethyl-tetrapalmityl spermine) and DOPE. The effects of divalent cations on the transfection with the newly devised liposome were investigated in detail. It is concluded that a mixture containing the PEI-1800 reagent provides a low cost and stable transfection procedure for the cultured silkworm cell line.

Key words: transfection, cationic lipid, cationic liposome, insect, cell culture

INTRODUCTION

Gene delivery is one of the important techniques in basic and applied biology. Cationic liposomes are commonly used for gene transfer in many cultured cell lines due to their convenience and efficiency. In general, the efficiency of transfection in insect cells using the non-viral gene transfer systems is lower than that in mammalian cells (Han, 1996). The maximum efficiency of gene transfection into a cell line derived from a lepidoptera, Bombyx mori, by lipofection is approximately 25% of total cell population (Keith et al., 2000), and the gene delivery by infection of a recombinant baculovirus has been favored in insect systems (Myles et al., 2001). This method has several advantages over the other transfection methods, very high efficiency, easy manipulation and low cost. Although the efficiency of baculovirus infection in lepidopteran cells is often as high as 100%, its infection causes not only arrest of cell proliferation but also many physiological changes coupled with viral gene expression in the host cells (Okano et al., 2001). To obtain cell lines which stably express foreign genes, this system requires extensive improvements.

Compared to the baculovirus system, DNA transfection using polycationic lipids or liposomes such as TM-TPS and DOPE was less effective; however, lipofection methods have been commonly used in other species. Various polycationic agents were developed for this purpose and were categorized into two groups, polycationic polymerized lipids and polycationic liposome containing at least one polycationic lipid. In polycationic polymer group, PEI is well known as a useful reagent for gene transfer. PEI has the highest cationic-charge density potential among the lipids used for gene delivery, and has been applied to ordinary gene transfer in gene therapy, due to its low toxicity for living cells, although the efficiency of DNA transfection using PEI is not so high (Boussif et al., 1995). Development of highly sensitive assays such as luciferase reporter system compensates the low efficiency of the non-viral gene delivery systems. Under some experimental conditions, however, a reporter gene must previously be introduced into the cells that are transfected with an effector gene. In this case the transfection efficiency using the non-viral systems is far from satisfaction, especially in insect cells. In order to overcome this problem, we have attempted to improve the efficiency of gene transfer and to establish an efficient PEI-mediated gene transfer procedure using a lepidopteran cell line.

MATERIALS AND METHODS

Cell line

Cells used were of the BmN4 cell line derived from B. mori, cultured and maintained in IPL-41 insect medium supplemented with 10% fetal bovine serum. Before use for transfection, this medium was replaced by Sf-900 SFMII serum free culture medium (Invitrogen).
Nucleic acids

The plasmid pSK8Fb-Luc, containing a firefly luciferase coding sequence under the control of a modified baculovirus ie-1 promoter (kindly provided by Professor Hisanori Bando of Hokkaido University Graduate School), was used for luciferase reporter assay. Another plasmid, pEXP38-βgal (Invitrogen), which contains the bacterial β-galactosidase gene under the control of the enhanced silkworm actin promoter (Lu et al., 1997), was used for β-galactosidase assay.

Lipid and liposome preparation

The polycationic lipids PEI 500kDa (PEI-500,000) was from Sigma, PEI 1800 Da (PEI-1800) and PEI 600 Da (PEI-600) were from Wako Chemicals. These were each dissolved in water to the concentration of 10 mg/ml, neutralized with HCl, filtered (Millipore, 0.2 μm) and stocked. CellFECTIN, a reagent comprising the polycationic lipids TM-TPS and DOPE at a ratio of 2 : 3 (1 mg/ml), was purchased from Invitrogen and used directly as liposome according to manufacturer’s protocol. DOTAP, a polycationic lipid (1 mg/ml) from Roche Diagnostics, was also used as liposome without further treatment. Other liposomes were prepared by the following procedures. Lyophilized DOT-AP from Sigma, DOPE from Wako and PEI 1800 Da were each dissolved in chloroform at a concentration of 10 mg/ml using siliconized tubes. These were mixed at various ratios, evaporated by aspirator to remove chloroform, purged vacuum bells by nitrogen gas, resuspended in deionized water at a concentration of 1 mg/ml, sonicated in a siliconized tube and stored at 4°C.

Transfection procedure

The plasmid (1 μg) to be tested was diluted into 10 μl with HEPES-buffered saline (HBS) and mixed on ice for 45 min with 20 μl aliquot of any of the liposome solutions, which were previously diluted into 20 μl with HBS. The mixture was allowed to stand for another 15 min, and overlaid onto the indicated number of cells. The suspension was incubated for 6 h, added with 1 ml of IPL-41, which was previously supplemented with 10% FBS, and incubated further. Luciferase assay was performed 36 h after the transfection, except for X-gal staining assay that was carried out 72 h after the transfection.

Luciferase assay

Expression of luciferase gene was monitored by using a Pica Gene luciferase assay kit (Toyo Ink). The cells transfected were washed three times with PBS, resuspended in 50 μl of lysis buffer (25 mM Tris-phosphate, pH 7.8, 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-N,N,N′,N′-tetraacetic acid, 10% glycerol and 1% Triton X-100) and incubated for 15 min. After centrifugation, 10 μl of cleared cell lysate was mixed with 20 μl of luciferase substrate solution (20 mM Tricine-NaOH, pH 7.8, 1.07 mM basic magnesium carbonate, 2.67 mM magnesium sulfate, 3.3 mM ethylenediaminetetraacetic acid, 33.3 mM dithiothreitol, 270 μM coenzyme A, 470 μM luciferin and 530 μM ATP in 1 × -PBS). The luciferase activity was measured using 1,253 Luminometer (Bio-Orbit). Each transfection was done in triplicate and is expressed as arbitrary units with SD. Overall experiments were repeated several times.

X-gal staining

Cell staining with β-galactosidase activity was performed as described (Ansubel et al., 1998). Briefly, the cells washed with PBS were fixed for 10 min in 0.25% glutaraldehyde, washed again and incubated in the dark (3 h at 37°C) in a buffer containing 1 mg/ml X-gal substrate, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 200 μM MgCl₂. The cells stained were observed by optical microscopy.

RESULTS

Comparison of various polycationic reagents and application of PEI as novel members of liposome

Among the commercially available cationic liposomes, CellFECTIN, i.e., TM-TPS : DOPE (2 : 3) was known to be useful regents for gene transfer in lepidopteran cells such as Spodoptela flugiperda derived cell lines, Sf-9 and SF-21. On the other hand, PEI, a highly charged cationic lipid, has been used in mammalian gene delivery systems (Boussif et al., 1995), especially in gene therapy due to its low cytotoxicity (Kircheis et al., 2002). To evaluate the transfection efficiency of these reagents, the luciferase expression plasmid was mixed with equal amounts of these reagents and introduced into the BmN4 cell line as described in MATERIALS AND METHODS (Fig. 1). The transfection by TM-TPS : DOPE (2 : 3) assayed as a reference was shown to be 1.4-fold more efficient than that using PEI-1800. In contrast, the transfection by PEI-500,000 or PEI-600 was resulted lower. In general, an excess amount of liposome caused a decrease of the efficiency of gene transfer (Gebhart et al., 2001). The transfection efficiency for PEI-1800 was compared by titrating the DNA to PEI-1800 ratio over a PEI-1800 amount range of 6 to 30 μg per sample. Then we determined 18 μg per experiment for 1 μg of DNA substrate (data not shown).

PEI-1800 was thus considered to be a useful reagent for transfection of insect cell lines, and, therefore, used to devise a novel liposome in combination with two kinds of lipids DOTAP, which is a commercially available cationic lipid, and DOPE, which is a fusogenic lipid commonly
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used as a helper lipid (Farhood et al., 1995) and often included in liposomal transfection reagents including the TM-TPS : DOPE. As shown in Fig. 2, the liposome formulated as PEI-1800 : DOTAP : DOPE at a 1:1:1 ratio (this liposome will hereafter be termed PDD111) increased the transfection efficiency by 2.5-fold that obtained by TM-TPS : DOPE (2 : 3). Under this experimental condition, the cytotoxicity of PDD111 measured by tetrazolium dye was as low as that of TM-TPS : DOPE (2 : 3) (data not shown). The helper lipid omitted liposome formulated as PEI-1800 : DOTAP at a 1:1 ratio was not increased it, and the mixture ratio of 1:1:2 was also decreased transfection efficiency (data not shown). The mixture at a ratio of 1:1:1 was found to give the maximum efficiency among different ratios tested. Then we determined 3 μg PDD111 per experiment for 1 μg of DNA substrate and used further study. The value obtained by PDD111 was about 4 times larger compared to that given by the liposome made, as reported in (Lampela et al., 2004), by mixing two-lipid liposome (DOTAP : DOPE) with PEI, even when the final ratio of the three lipids also adjusted to 1:1:1 (Fig. 2, the last column).

Effects of divalent cations on the efficiency of lipofection

The effects of five divalent cations, Mg$^{2+}$, Ca$^{2+}$, Mn$^{2+}$, Zn$^{2+}$ and Sr$^{2+}$ on the overall transfection efficiency were examined (Fig. 3A, C and E). In other series of experiments, the divalent cations were added after the mixing of plasmid and liposome in order to reduce the effects of divalent cations on the DNA-liposome complex formation (Fig. 3B, D and F). Mg$^{2+}$, Ca$^{2+}$ and Sr$^{2+}$ have no effect on the PEI-1800-mediated overall reaction at all concentrations examined (Fig. 3A); the increasing concentrations of Zn$^{2+}$ and Mn$^{2+}$ decreased the efficiency. When the cations were added after the complex formation, up to 2.5-fold increases in the reporter activities were detected at the Zn$^{2+}$ concentrations of 1.6 mM (Fig. 3B). Ca$^{2+}$ at the concentrations of 0.32 to 16 mM increased the efficiency of overall complex formation with TM-TPS : DOPE (2 : 3) (Fig. 3C). Sr$^{2+}$ indicated the same tendency. Zn$^{2+}$ at the concentration of 0.32 mM drastically increased the efficiency. Different effects of Ca$^{2+}$ or Sr$^{2+}$ in comparison to Fig. 3C were seen when these were added after the complex formation (Fig. 3D), suggesting that these divalent cations might act rather on the interaction between DNA and the liposome. With respect to PDD111, the overall reaction was slightly stimulated only by the addition of 32 mM Mg$^{2+}$ (Fig. 3E). The addition of 32 mM Mg$^{2+}$ or Ca$^{2+}$ to the PDD111 liposome after the complex formation slightly increased the transfection efficiency (Fig. 3F). The finding that transfection efficiency of liposome containing DOPE was enhanced by the presence of Ca$^{2+}$ or Sr$^{2+}$ as seen in Fig. 3C are in agreement with previous studies (Kaighn et al., 1989; Lam et al., 2000), but, to our knowledge, the detailed effects of cations on the efficiency of PEI-mediated transfection was first reported in this study. In comparison with PEI-1800 alone or by TM-TPS : DOPE (2 : 3), the currently devised lipofectin PDD111 showed smaller effects by the addition of divalent cations upon the transfection efficiency.

Fig. 1. Comparison of lipofection efficiency among polyethylenimine (PEI) preparations with various molecular weights. TM-TPS : DOPE (3 : 2) was also assayed as a reference. BmN4 cells (2 × 10$^5$ cells in 24-well multi plates) were transfected with 1 μg pSK8-Fbluc as detailed in MATERIALS AND METHODS. All liposome reagents were each used in 6 μg for transfection. After 6 h post transfection, medium was replaced IPL-41. Luciferase activity was assayed after 36 h.

Fig. 2. Comparison of lipofection efficiency of PDD111 with other liposomes. PDD111 is a liposome newly formulated as DOTAP : DOPE : PEI in equal amount. TM-TPS : DOPE (3 : 2) was assayed as a reference. DOTAP : DOPE + PEI was also a complex liposome wherein a preformed mixture DOTAP : DOPE (1 : 1) was further mixed with PEI-1800. For other details see the legend to Fig. 1.
Comparison of the transfection efficiency by X-gal staining

All of the above-indicated transfection efficiency data were evaluated by using luciferase luminescence as a reporter. However, it is not clear that the value of luciferase activity is strictly related to the cell population that expresses the reporter protein. In order to clear this point, we compared the transfection efficiency by X-gal staining using pEXP38-βgal expressing β-galactosidase under the control of B. mori actin delived promoter (Lu et al., 1997). The PDD111 liposome showed a similar transfection potency (approximately 40% positive) to TM-TPS : DOPE in terms (approximately 35% positive) of cell population expressing β-galactosidase, but much better than DOTAP (approximately 5% positive) (Fig. 4).

DISCUSSION

In this report, we improved the transfection technique for cultured lepidopteran cells using a lipid- or liposome-mediated gene transfer system. To this technique, the selection of cationic lipid or liposome is very important, and the binding of nucleic acid to lipid or liposome show critical effects for transfection efficiency (Dias et al., 2002). We have demonstrated that PEI is a useful transfection reagent for the cells as a liposome. PEI is an organic macromolecule with a very high cationic-charge-density potential and have two advantages over other transfection reagents. One is that the efficiency of transfection is relatively independent from the phase of cell cycle (Brunner et al., 2001). Another is that nucleic acid bound by PEI is protected from nuclease degradation (Dheur et al., 1999; Ferrari et al., 1999). We compared several commercially available PEIs in our experiments, and found that the most effective PEI was different from those fitted for mammalian cells (Boussif et al., 1995). It may be due to the difference in lipid composition of the cell surface. The polycationic lipid mixture, PDD111 (DOTAP : DOPE : PEI-1800 at a 1:1:1 ratio), developed in the present study gave high transfection efficiency on the luciferase and β-galactosidase reporter systems. Using PDD111 we examined the effects of several parameters on the efficiency of transfection. Since it is known that Ca\textsuperscript{2+} and Sr\textsuperscript{2+} enhance transfection efficiency, we examined the effects of these cations, as well as of other diveral cations. Ca\textsuperscript{2+} and Sr\textsuperscript{2+} seem to be similar to each other with respect to the behavior at a complex formation between the regents and the plasmid, but showed enhancements only on

Fig. 3. Effects of divalent cations on complex formation and on transfection. For overall transfection, 1 µg pSK8-Fblue was complexed with 18 µg of PEI-1800, 6 µg of TM-TPS : DOPE (2 : 3) or 3 µg of PDD111 in the presence of indicated concentrations of divalent cations (A, C and E). To reduce the effects of divalent cations on the plasmid-liposome interaction, the divalent cations were added to the mixture after the complex formation (B, D and F). A and B, PEI-1800; C and D, TM-TPS : DOPE (2:3); E and F, PDD111. Divalent cation concentrations in the final cell culture are indicated.

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DOPE-containing liposomes. These results were in agreement with those obtained in a previous study (Lam et al., 2000). The function of these divalent cations during the complex formation remains unclear, but Ca$^{2+}$ combined within the complex is possible to enhance endocytotic uptake of DNA (Lam et al., 2000). Sr$^{2+}$ may be a calcium analogue on transfection (Kaighn et al., 1989), since it is an alkaline earth metal, to which group Ca$^{2+}$ also belongs, and likely to behave in solution similarly to Ca$^{2+}$.

The contribution manner of Zn$^{2+}$ for the transfection efficiency is different from those of Ca$^{2+}$ and Sr$^{2+}$, since Zn$^{2+}$ is less effective to the overall transfection processes but effective on the step after the liposome-DNA complex formation. Also the concentrations of Zn$^{2+}$ required for the effective enhancements were different from the above divalent cations. These observations suggested that Zn$^{2+}$ improves general endocytosis, not specific-uptake of DNA-liposome-cation complex. Previous studies have reported that Zn$^{2+}$ is effective for endocytosis (Chang et al., 1998) and functions differently from Ca$^{2+}$ (Tros de Ilarduya and Duzgunes, 2000).

Compared to commercially available liposome, such as TM-TPS : DOPE (2 : 3) or DOTAP, the transfection using the PEI-containing liposome PDD111 (also comprises DOTAP and DOPE) was successful in both luciferase and β-galactosidase reporter systems, indicating that the liposome delivers DNA to large population of cells, not by increasing the amount of DNA introduced into a cell. This inference is further supported by the possibility that DOTAP and PEI have different attachment sites for cell surface. Therefore, the liposome containing DOTAP and PEI may be utilized to introduce DNA into cells under different physiological conditions, such as cell cycle phase. Also, it is anticipated that this reagent will be useful for effective transfection with low cytotoxicity. The transfection efficiency of PDD111 did not profoundly affect in the presence of various cations than other liposomes. Additionally, our preliminary data demonstrated that the PDD111 could be used to introduce DNA into other insect cell lines, such as Sf9 of lepidopteran cell line and S2 of dipteran cell line (data not shown).

X-gal stain indicated that transfected cell population using PDD111 was similar to that using TM-TPS : DOPE (2 : 3). However, expression levels of luciferase gene were quite different between these liposomes. Combining these results, we suggest the PDD111 may well be delivering more DNA to the cells than TM-TPS : DOPE (2 : 3). This phenomenon may be due to PEI characteristics. When transferred into the cytoplasm, PEI protects DNA from damage and endosomal degradation when complexed with PEI (Marshall et al., 1995). Perhaps condensed DNA binds less strongly to cellular components than the complex with TM-TPS : DOPE, and less restricted for the movement through the cytoplasm.

ACKNOWLEDGEMENTS

We are grateful to Dr. K. Kojima and Professor H. Bando of Hokkaido University Graduate School for supplying the plasmid, pSK8Fb-Luc. This work was supported in part by grants (Nos. 14704010 and 14656024) and National Bioresource Project from the Ministry of Education, Science and Culture of Japan.

REFERENCES


