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Synergistic effect of polyethylenimine and cationic liposomes in nucleic acid delivery to human cancer cells

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Abstract

Polyethylenimine (PEI) and other polycations are good vehicles for transferring genes into the cells. In earlier reports, poly-L-lysine and protamine have been shown to improve gene delivery with cationic liposomes. In this study, PEI, combined with different cationic liposomes, was studied to determine the optimal conditions for gene delivery. The reporter genes, luciferase and green fluorescent protein, were used to transfect human HeLa, HepG2 and hepatoma 2.2.15 cells with various combinations of PEIs (0.8 and 25 kDa), poly-L-lysine (15–30 kDa), protamine and cationic liposomes. The highest expression level was achieved by using the combination of PEI 25 kDa (0.65 μg/μg of DNA, nitrogen-to-DNA phosphate (N/P) ratio = 4.5) with 10 nmol of DOTAP–cholesterol (DOTAP–Chol, 1:1 w/w). This DNA complex formulation dramatically increased the luciferase expression 10- to 100-fold, which was much higher than those of other polycations alone, cationic liposomes alone or the combination. In addition, PEI/DOTAP–Chol combination had little cytotoxicity than DOTAP–Chol or other cationic liposomes alone. The effect of oligonucleotide (ODN) delivery facilitated by PEI and cationic liposomes was also studied in the hepatoma cell lines. We demonstrated an antisense ODN of p53 delivered by PEI/DOTAP–Chol combination effectively inhibited the biosynthesis of p53 protein in HepG2 (68% inhibiton) and 2.2.15 cells (43% inhibition). Thus, the large PEI could synergistically increase the transfection efficiency when combined with the cationic liposomes.

Keywords: Polyethylenimine; Polycation; Cationic liposome; DNA transfection; Oligonucleotide; Nonviral vector

1. Introduction

Liposomes and polycations are the two major classes of nonviral gene delivery methods in vitro and in vivo [1–9]. Significant limitations of cationic liposomes are low transfection efficiency and unable to transfect any cells, especially the hepatoma cells. In order to solve the problems, the discovery of new lipids and new helper compounds is currently underway to improve the transfection efficiency of liposomes. The addition of polycations, i.e. poly-L-lysine and protamine, as co-polymer can markedly enhance the transfection efficiency of several types of cationic liposome by 2–28-fold in a number of cell lines in vitro [10] and in vivo [11].

Polyethylenimine (PEI) has different molecular weights associated with different transfection efficiencies [8]. The ability of DNA condensing properties and proton-sponge effect of PEI are important factors for gene delivery [8,12], whether genes may be delivered into cytoplasm via endocytosis or targeted to the nucleus [13]. Only the PEIs larger than 10 kDa have been used successfully in transfection studies [8,14]. In them, the linear PEI of 22 kDa [15] and the branched PEI of 25 kDa [16] are the most effective transfection reagents used in vitro and in vivo.

Low-molecular-weight PEIs (average MW of 0.6 and 1.8 kDa) are ineffective in terms of gene transfer [17]. However, if combined with cationic or anionic liposomes, such low-molecular-weight PEIs can be effective to deliver the genes into cells [18–22]. This effect may be due to the synergistic advantages of both PEI and cationic liposomes. On the other hand, the effectiveness of large PEI, such as 25 kDa, is controversial as a good co-polymer in the combined use for gene delivery [19,21,22].

We are interested in elucidating whether the combination of PEIs and cationic liposomes enhances the transfection efficiency in human cancer cells. We tested the different

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cationic liposomes and polycations in the combination system for plasmid and oligonucleotide (ODN) delivery in vitro, and aimed to optimize gene transfection condition.

2. Materials and methods

2.1. Materials

1,2-Dioleoyl-3-(trimethylammonium) propane (DOTAP), dimethyl dioctadecyl ammonium bromide (DDAB), 1,2-dioleoyl-3-*sn*-phosphatidyl-ethanolamine (DOPE) and cholesterol were from Avanti Polar Lipids (Alabaster, AL, USA). Polylysine (PLL) (15–30 kDa), protamine and PEIs of different molecular weights (0.8 and 25 kDa) were obtained from Aldrich-Sigma (St. Louis, MO, USA). Opti-MEM I medium was from Gibco/BRL (Carlsbad, CA, USA). Plasmid Maxiprep Kit was from Qiagen (Hilden, Germany). Luciferase assay kit was purchased from Promega (Madison, WI, USA). DC Protein Assay reagent was from Bio-Rad (Hercules, CA, USA). The 18-mer phosphorothioated antisense of p53 ODNs (246-CGGCTCCTCCATGGCAGT-263) and sense (263-ACTGCCATGGAGGAGCCG-246) were synthesized on a PE Applied Biosystems DNA synthesizer. The sense ODNs of p53 were used as negative control. Anti-p53 monoclonal antibodies were purchased from R & D Systems (Minneapolis, MN, USA). Anti-β-actin and secondary HRP-conjugated antibodies were from Aldrich-Sigma.

2.2. Cell cultures

Human HeLa, HepG2 and hepatoma 2.2.15 cells, which are of hepatitis B virus (HBV) DNA transfected human hepatocarcinoma cell line [23], were purchased from ATCC (Maryland, USA). All cell lines were cultured in DMEM with high glucose (DMEM-HG) supplemented with 10% (v/v) heat-inactivated fetal bovine serum. These cells were maintained at 37 °C, under 5% CO_2. For transfection, 1×10^5 cells were initially seeded in 1 ml of medium in 12-well culture plates and transfected at cell density of 70% confluency.

2.3. Plasmid preparations

pCMV-EGFP and pCMV-Luc, encoding the green fluorescent protein and firefly luciferase, respectively, with human cytomegalovirus promoter, were amplified in DH5α strain of *E. coli*, and purified using the Qiagen Plasmid Maxiprep Kit (Chatsworth, CA, USA).

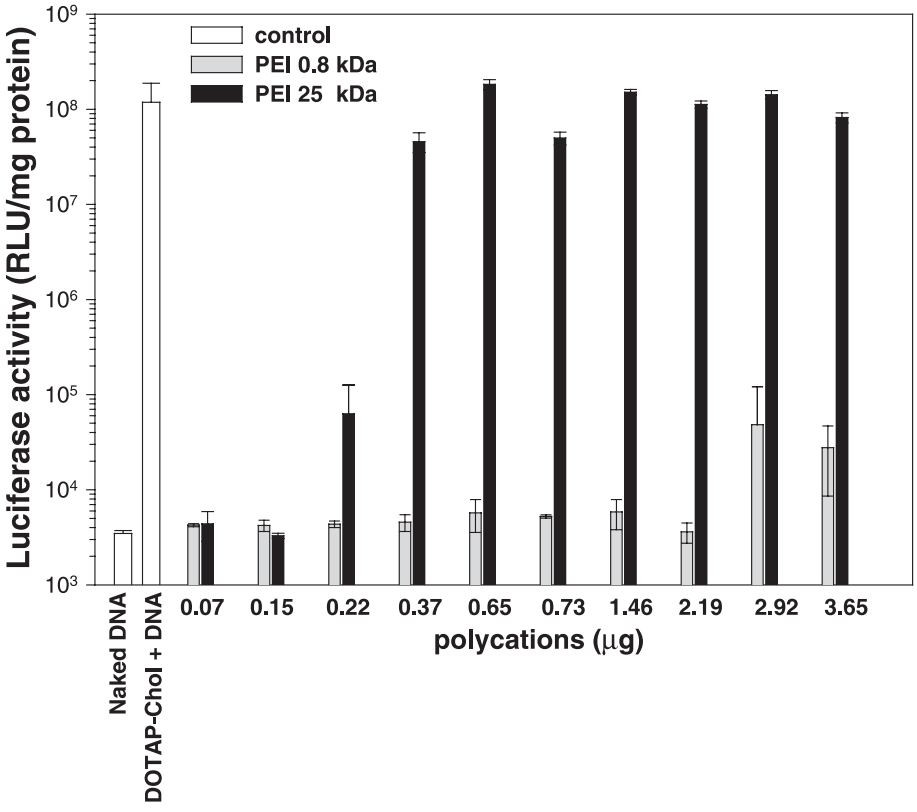

Fig. 1. Effect of different PEIs on the transfection efficiency in HeLa cells. The cells were transfected with 1 μg of pCMV-Luc plasmid complexed with PEI 0.8 or 25 kDa at ratios (w/w) from 0 to 3.65 (N/P ratio equivalent to 0–25) or with 10 nmol of DOTAP–Chol/DNA. The cells were incubated in the transfection solution for 1 h and then in the growth medium for 24 h before the luciferase assay. These data are expressed as relative light unit (RLU) of luciferase activities per milligram of total cell protein (mean ± standard deviation, obtained from triplicate experiments). The experiment was repeated three times with similar results.

2.4. Liposome preparations

DOTAP, DDAB, DOPE and cholesterol were dissolved in chloroform, sealed in ampoules filled with argon gas and stored at −20 °C. Small unilamimellar vesicles (SUV) were prepared by a combination of the standard thin-film hydration method and repeated extrusion as described elsewhere [24]. Briefly, cationic lipids with DOPE or cholesterol (molar ratio 1:1) were dissolved in chloroform and placed in a round-bottomed flask. The solvent was removed by rotary evaporation under reduced pressure. The lipid film was hydrated in 5% dextrose solution to give a final concentration of 10 μM cationic lipids per milliliter. The lipid solution was sequentially extruded through polycarbonate membranes, three times at pore size of 0.1 μm and seven times at 0.05 μm by using high-pressure extrusion equipment from Lipex Biomembranes (Vancouver, BC, Canada) at 55 °C. The cationic liposomes were filter-sterilized through Millex 0.22-μm-diameter filter and stored at 4 °C in argon gas before use.

2.5. Preparations of nucleic acid/polycation/liposome complexes

DNA of pCMV-Luc, pCMV-EGFP, sense or antisense p53 ODNs were mixed with polycations (PLL, protamine or PEI) of different weight ratios in 50 μl of HBS buffer (a solution of 100 mM NaCl, 20 mM Hepes, pH 7.4). Lipoplexes were prepared by gently mixing 10 nmol of liposomes (based on the level of cationic lipids) in 50 μl of HBS buffer with 1 μg of ODNs, pCMV-Luc or pCMV-EGFP plasmid in 50 μl of HBS buffer with or without polycations. After incubation for 15 min at room temperature, the DNA/polycation/liposome complexes were added to the culture cells for transfection. Either luciferase activity or GFP expression on these cells was examined (see below).

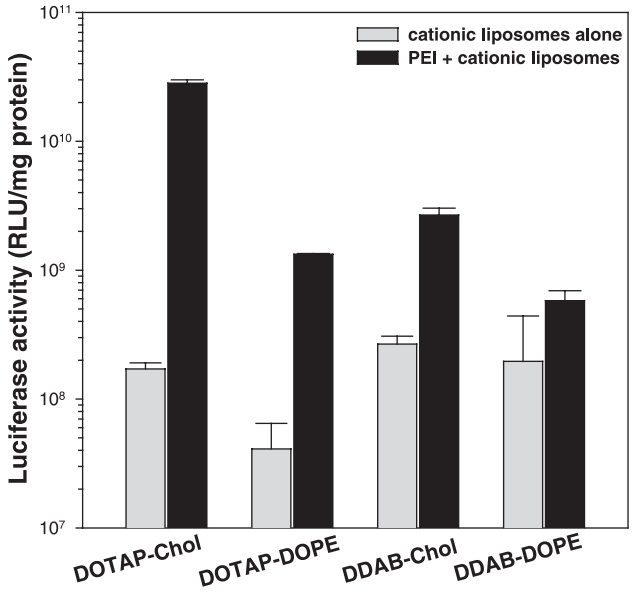

Fig. 3. Effect of different cationic lipids on the transfection efficiency of PEI/cationic liposomes combinations in HeLa cells. For the complex formulation, 1 μg of pCMV-Luc plasmid was mixed with 10 nmol of different cationic liposomes indicated, in the presence or absence of 0.65 μg of PEI 25 kDa. The cells were incubated in the transfection solution for 1 h and then in the growth medium for 24 h before the luciferase assay. These data are expressed as relative light unit (RLU) of luciferase per milligram of total cell protein (mean ± standard deviation, obtained from triplicate wells). The experiment was performed three times with similar results.

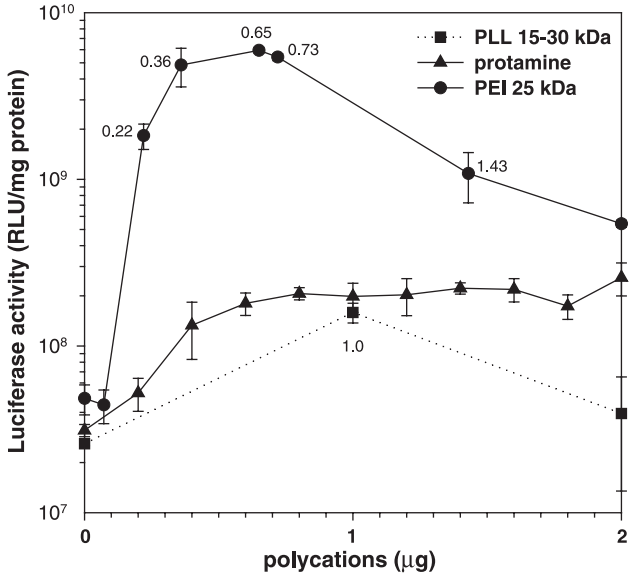

Fig. 2. Effect of different polycations/DNA and DOTAP–Chol ratios on the transfection efficiency in HeLa cells. The cells were transfected with 1 μg of pCMV-Luc plasmid with 10 nmol of DOTAP–Chol complexed with or without 0–2 μg of PLL (15–30 kDa), protamine and PEI 25 kDa. The cells were incubated in the transfection solution for 1 h and then in the growth medium for 24 h before the luciferase assay. These data are expressed as relative light unit (RLU) of luciferase activities per milligram of total cell protein (mean ± standard deviation, obtained from triplicate experiments). The experiment was performed three times with similar results.

2.6. Luciferase assay

Cells were seeded in the 12-well culture plates 1 day before transfection. The culture medium was replaced with 0.9 ml of OPTI-MEM I medium before adding 0.1 ml of DNA/polycation complex, DNA/liposome complex or DNA/polycation/liposome complexes. After incubation for 1 h (in 5% CO_2 at 37 °C), 1 ml of medium with 10% of serum was added and incubated for another 24 h. On the day of harvest, cells were washed with PBS twice and then lysed with buffer containing 150 mM PBS, pH 7.4, 1% Triton X-100, 1 mM dithiothreitol and 2 mM EDTA. Luciferase activities in the lysates were evaluated by using the Monolight Luminometer 2010 from Becton-Dickinson (San Jose, CA, USA). Protein content was measured by DC Protein Assay kit with bovine serum albumin as standard. The data

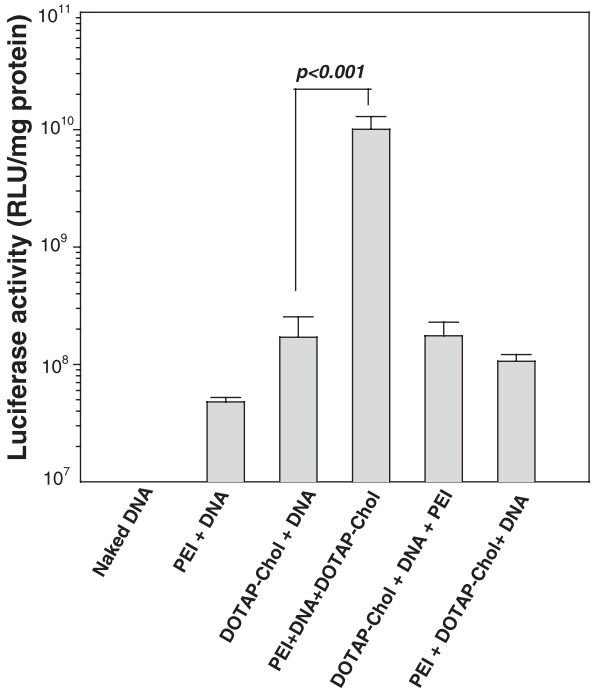

Fig. 4. Effect of different combinations of the transfection reagents in HeLa cells. The transfection complexes were composed of 1 μg of pCMV-Luc plasmid with the choice of DOTAP–Chol (10 nmol) and PEI 25 kDa (0.65 μg). The mixing procedures to form the DNA complexes were indicated in the abscissa. HeLa cells were incubated in the transfection solution for 1 h and then in the growth medium for 24 h before the luciferase assay. These data are expressed as relative light unit (RLU) of luciferase activities per milligram of total cell protein (mean ± standard deviation, obtained from triplicate wells) ($P < 0.001$). The experiment was repeated three times with similar results.

were expressed as relative light unit (RLU) of luciferase per milligram of total cellular protein. For the normalization, 1 ng of luciferase protein could generate 2000 RLU in our luciferase activity assay.

2.7. Flow cytometry and fluorescent imaging analysis

The pictures of GFP-transfected cells were taken under the fluorescent microscope after 24 to 48 h of incubation. For flow cytometry, hepatoma cells after transfected with pCMV-EGFP were washed with PBS and trypsinized. Transfection efficiency was evaluated by scoring the percentage of cells expressing green fluorescence protein using a FACS Calibur System from Becton-Dickinson. The experiments were performed in triplicates and 10,000 cells were count in each experiment.

2.8. Western blot analysis

Cells were lysed in a buffer composed of 150 mM NaCl, 50 mM Tris (pH 8.0), 5 mM EDTA, 1% (v/v) nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin and 25 μg/ml leupeptin at 4 °C. Equal amounts of protein extracts were resolved by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose filter. After blocking with buffer containing 20 mM Tris–HCl (pH 7.5) and 150 mM NaCl, 5% nonfat milk for 1 h at room temperature, the filter was incubated with specific antibodies against p53 and β-actin for 1 h at room temperature followed by the use of horseradish peroxidase-labeled secondary antibodies. Blots were developed using a chemiluminescent detection system form Amersham Biosciences UK Ltd. (Little Chalfont, Buckinghamshire, UK). Protein bands on the film were quantified by scanning with ImageMaster densitometer from Amersham Pharmacia Biotech (Piscataway, NJ, USA).

3. Results

3.1. Effect of different PEIs on gene delivery in HeLa cells

The transfection abilities of PEIs (MW 0.8 and 25 kDa) and DOTAP–Chol were compared in this experiment. The cells were transfected with 1 μg of pCMV-Luc plasmid complexed with PEI 0.8 or 25 kDa from 0 to 3.65 μg (N/P ratio equivalent to 0 to 25) or with 10 nmol of DOTAP–Chol/DNA. When PEI/DNA weight ratio ≥ 0.37, the transfection efficiency of PEI 25 kDa was 1000-fold more effective than that of PEI 0.8 kDa in the HeLa cells. The

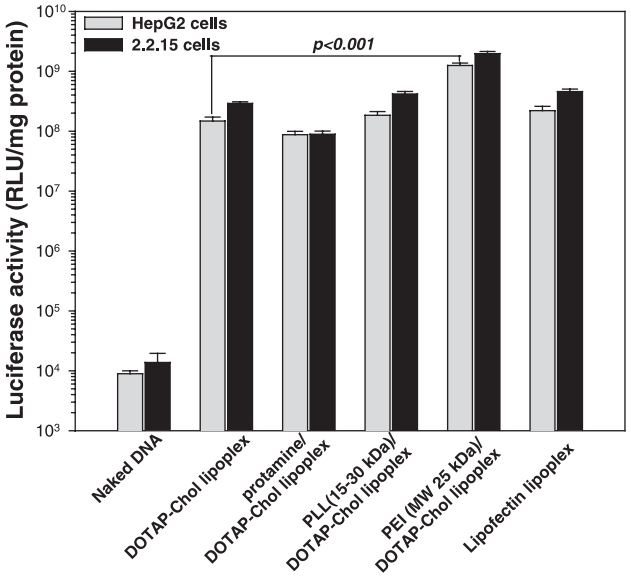

Fig. 5. Effect on the transfection efficiency of polycation/DOTAP–Chol combinations in liver cell lines. The human hepatoma HepG2 and 2.2.15 cells were transfected with 1 μg of pCMV-Luc plasmid combined with the choice of PEI 25 kDa (0.65 μg), PLL (1.0 μg) and protamine (0.8 μg) before adding DOTAP–Chol (10 nmol). The cells were incubated in the transfection solution for 1 h and then in the growth medium for 24 h before the luciferase assay. These data are expressed as relative light unit (RLU) of luciferase activities per milligram of total cell protein (mean ± standard deviation, obtained from triplicate wells). Three independent experiments were performed with similar results.

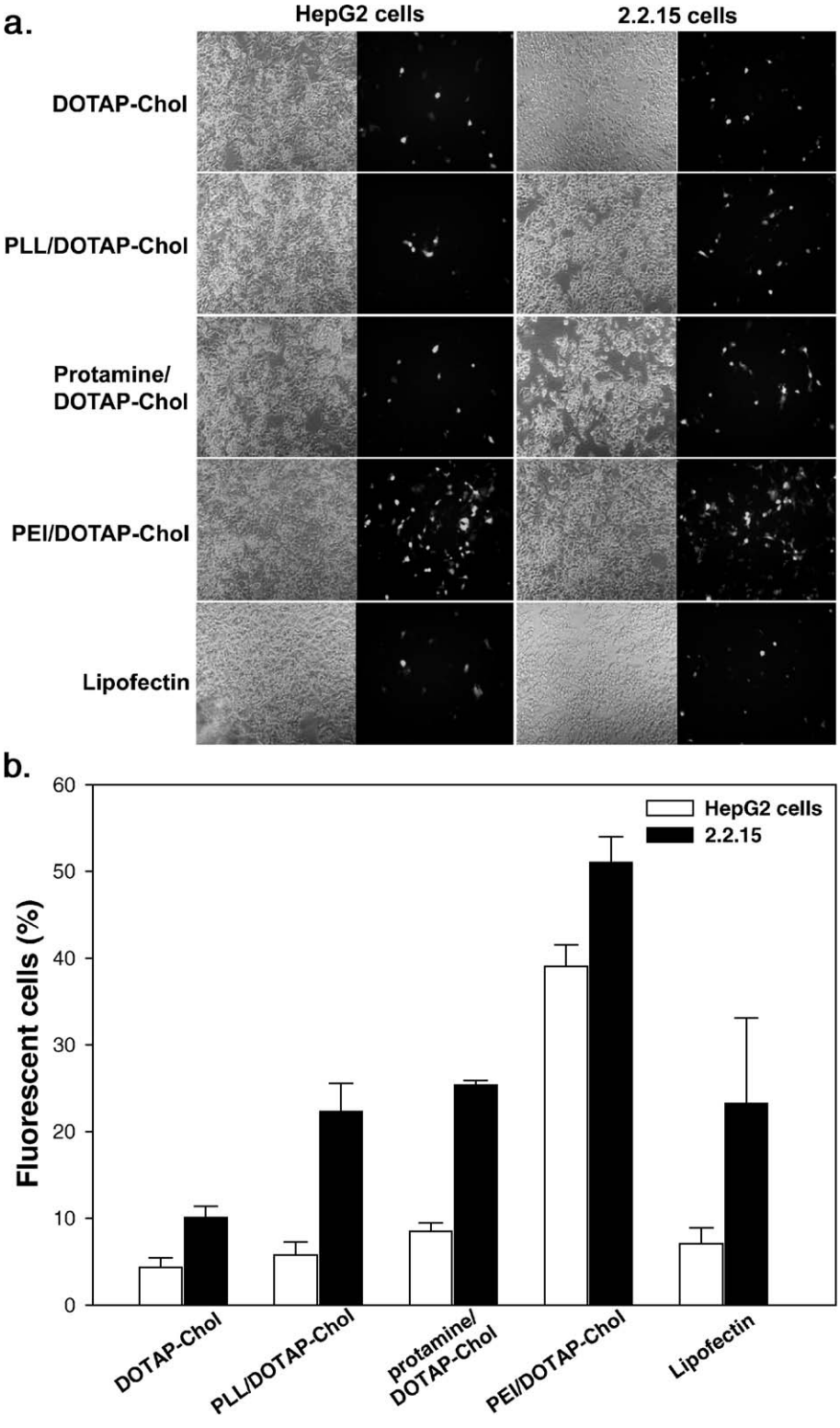

Fig. 6. Effect of polycation/DOTAP–Chol combinations on the cell transfection. Human hepatoma HepG2 and 2.2.15 cells were transfected with 1 μg of pCMV-EGFP plasmid complexed with PLL, protamine or PEI 25 kDa at optimal weight ratio before adding 10 nmol of DOTAP–Chol. Cationic liposomes such as Lipotectin and DOTAP–Chol served as control. The cells were incubated in the transfection solution for 1 h and then incubated in growth medium for another 48 h. After incubation, the cells were washed with PBS and fixed with 4% paraformaldehyde for 15 min. Then the efficiency of transfected cells was determined by fluorescence microscopy (a). For flow cytometry, hepatoma cells after transfection with pCMV-EGFP were washed, trypsinized and quantified using the FACS machine as described in the Materials and methods (b).

effectiveness of PEI 25 kDa was similar to DOTAP–Chol complexes in this transfection experiment (Fig. 1).

3.2. The role of polycations in liposome-mediated gene delivery in HeLa cells

The cells were transfected using PEI 25 kDa, PLL or protamine combined with DNA and DOTAP–Chol. In the presence of 10 nmol of DOTAP–Chol, the best luciferase activity was achieved at a PEI/DNA weight ratio of 0.65, PLL/DNA weight ratio of 1.0 and protamine/DNA weight ratios of 0.8–2.0 (Fig. 2). The addition of polycations irrespective of PEI, PLL or protamine to DOTAP–Chol enhanced the transfection efficiency 10- to 100-fold. Additionally, we used the hemocytometer for the assessment of cell toxicity. There was minimal cytotoxicity as cells were treated with polycation/DOTAP–Chol combination (data not shown).

3.3. The transfection efficiency of PEI on gene delivery was combined with different cationic liposomes in HeLa cells

The different cationic liposomes were combined with PEI 25 kDa for gene delivery in HeLa cells. DOTAP and DDAB were individually formed liposomes with cholesterol or DOPE as helper lipids. The highest expression level was achieved by using the combination of PEI (0.65 μg/μg of DNA, N/P ratio = 4.5) with 10 nmol of DOTAP–cholesterol (DOTAP–Chol, 1:1 w/w) (Fig. 3).

3.4. Effect of different combinations with polycation- and liposome-mediated gene transfection in HeLa cells

To determine the best mixing procedure to form the DNA complexes, we added PEI 25 kDa and DOTAP–Chol to the plasmid DNA in different orders. The best transfection efficiency was achieved when PEI and DNA were mixed first, and then added DOTAP–Chol (Fig. 4).

3.5. The use of polycations/DOTAP–cholesterol combination in the gene delivery to human hepatoma cells

In order to see whether the combination of DOTAP–Chol and polycations can be used in other cells, we performed our studies using hepatoma HepG2 and 2.2.15 cells. In the case of HeLa cells, the best formulation was achieved with DNA/PEI/DOTAP–Chol at 1 μg:0.65 μg:10 nmol (Fig. 2). In adapting this combination to transfect HepG2 and 2.2.15 cells, we found that it worked as good as in HeLa cells and was 10 times better than Lipofectin in both cell lines (Fig. 5).

3.6. Effect of the polycations/DOTAP–Chol combination on the transfection of GFP reporter genes in human hepatoma cells

The transfection efficiency was counted by green fluorescence protein (GFP) expression in this experiment by using combinations of polycations and cationic liposomes. When DOTAP–Chol was added to the PEI/DNA complexes, the number of green fluorescent cells increased, compared with DNA/PLL/DOTAP–Chol, DNA/protamine/DOTAP–Chol, Lipotectin and DOTAP–Chol alone (Fig. 6a). These results showed that GFP expression was maximized in hepatoma HepG2 and 2.2.15 cells at DOTAP–Chol/DNA ratio (nmol/μg) of 10 combined with 0.65 μg of PEI 25 kDa. The percentage of green fluorescent-positive cells was about 50% (hepatoma 2.2.15) and 40% (HepG2) by FACS analysis. These expression levels were 10 times better than the use of DOTAP–Chol alone (Fig. 6b).

3.7. Use of PEI with DOTAP–cholesterol combination in the delivery of antisense ODNs to hepatoma cells

In order to determine whether PEI 25 kDa and/or DOTAP–Chol could deliver ODNs into cells, the p53 antisense ODNs were used as a surrogate. Hepatoma cells were treated with PEI/DOTAP–Chol containing p53 antisense ODN at a liposome/DNA ratio of 10. Both sense ODN and antisense ODN alone had no effect in decreasing the p53 protein production (Fig. 7). The inhibitory percentage of p53 protein expression in HepG2 cells was 68% as using the PEI/DOTAP–Chol formulation. Similarly, p53 protein was also reduced 43% in the treated hepatoma 2.2.15 cells. As seen in these results, PEI combined with DOTAP–Chol had a better delivery of ODN than DOTAP–Chol alone.

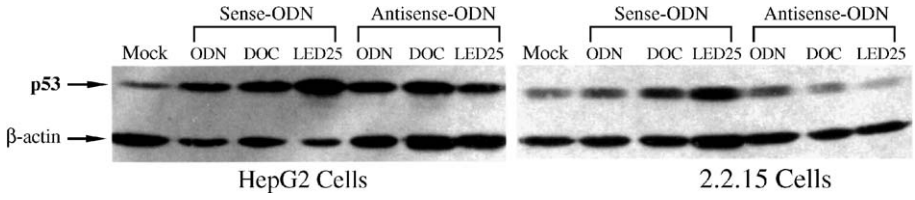

Fig. 7. Western blot analysis of the inhibition of p53 protein expression by PEI/DOTAP–Chol combination. One microgram of sense or antisense ODN was delivered into hepatoma HepG2 and 2.2.15 cells using 10 nmol of DOTAP–Chol with or without 0.65 μg of PEI 25 kDa. DOTAP-Chol formulation is indicated as DOC in this Figure, where as PEI 25 kDa/DOTAP-Chol formulation is indicated as LED25. β-actin was used as an equal loading indicator. The experiment was performed four times with similar results. Protein bands on the film were quantified by scanning with ImageMaster densitometer. The p53 protein was normalized with β-actin in the same gel.

4. Discussion

Nonviral DNA delivery is a multistage process involved in DNA packaging, cell entry, endosomal escape, nuclear localization and gene expression. These gene delivery vehicles usually consist of cationic polymers (polycations) or cationic liposomes. PEI is a synthetic polymer and has various molecular weights with different efficiencies for gene delivery [8]. The DNA condensing property and proton-sponge effect are the characteristics of PEI as a superior transfection reagent.

In this paper, a synergistic effect on the transfection efficiency was clearly demonstrated by the combined use of polycations and cationic liposomes. The highest expression level was achieved by using the combination of PEI 25 kDa (0.65 μg/μg of DNA, N/P ratio = 4.5) with 10 nmol of DOTAP–Chol. This formulation dramatically increased the luciferase or GFP expression 10- to 100-fold, which was much higher than those of other polycations alone, cationic liposomes alone or the combination. The optimal ratio of PEI/DOTAP–Chol complexes that works well in HeLa cells can be applied to liver cells, including hepatoma Huh7, Hep3B (data not shown), HepG2 and 2.2.15 cells. Since a complete condensation of DNA occurred at higher N/P ratio [20], the synergism appeared when the plasmid DNA was partially complexed but not fully condensed with PEI before adding the DOTAP–Chol (Figs. 2 and 4). Furthermore, it was proposed that DOPE promotes the endosomal membrane fusion and allows the DNA release to the cytoplasm. Therefore, DOPE is frequently formulated in the commercially available transfection reagents. But from our long-term experiences, the cationic liposomes containing cholesterol, e.g. DOTAP–Chol, worked slightly better than the one containing DOPE in the transfection of cultured cells. The adding of PEI (N/P ratio less than 5) can further enhance the cationic liposome-mediated gene delivery in these experiments. It suggested that the conformation of DNA complex, the proton-sponge effect and the membrane fusion mechanism are important factors and synergistically contributed to the gene transfection event [25,26].

Many polycations (PLL 4 kDa, PLL 20 kDa, PLL 200 kDa, PEI 25 kDa, PEI 800 kDa and dendrimer) and cationic liposomes alone are not effective carriers for antisense ODN [27,28]. In this study, we used the antisense ODN of p53 to inhibit the p53 protein expression, as measured by immunoblotting with anti-p53 monoclonal antibodies [29]. A significant decrease in p53 protein biosynthesis in HepG2 and hepatoma 2.2.15 cells was only seen with PEI/DOTAP–Chol formulation but not with cationic liposomes or PEI alone. In addition to the small PEIs [22,30], we used the large-molecular-weight PEI to achieve the sufficient transfection efficiency in this experiment. The nucleic acid condensation by PEI/DOTAP–Chol could help the ODN stably associated with the carriers, and would facilitate the ODN release into the cytoplasm. To our knowledge, this paper is the first one to describe the use of small amount of PEI combined with cationic liposomes for the delivery of plasmid DNA and ODNs to human cancer cells.

Moreover, we have found low toxicity in HeLa cells, even at the high liposome/DNA ratio of 10 when combined with PEI. The cell survival was at least 80%, which was similar to the reports using PEI 50 and 800 kDa [31]. The combination of PEI allowed us to use lower amount of liposomes, which would be an advantage because excess amount of cationic liposomes could cause cytotoxic effect.

In summary, the combination of PEI and DOTAP–Chol caused more than 10-fold increases in the transfection efficiency in many cells compared with using polymer or liposome alone. A minimal toxicity was also shown to these cultured cells in vitro. These data on PEI/DOTAP–Chol delivery system suggested that this formulation is very promising in delivering nucleic acids including plasmid DNA, RNA and ODNs into cells. The further understanding of the detail mechanism of the PEI/liposome-mediated synergism is mandatory to improve the design of future gene transfer tools.

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