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Evaluation of Transferrin-Polyethylenimine Conjugate for Targeted Gene Delivery

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With the aim to improve the specificity and to reduce the cytotoxicity of polyethylenimine (PEI), we have synthesized the conjugates of the branched PEI (25 kDa) with transferrin. The transferrin-PEI (TP) conjugates with five compositions were synthesized using periodate oxidation method and confirmed by FT-IR spectroscopy and gel permeation chromatography. The free amine contents of TP conjugates, which were able to condense and deliver DNA, increased as the amount of PEI increased. TP/DNA polyplexes were characterized by measuring gel electrophoresis, ethidium bromide fluorescence quenching, particle size and zeta potential of complexes. Complete complexation of the polyplexes was observed above the N/P ratio of 5 in TP/DNA, and above 3 in PEI/DNA, respectively. The zeta potential of the complexes decreased as the amount of transferrin in TP conjugates increased. Transfection efficiency of TP conjugates was evaluated in HeLa cell and Jurkat cell systems. Among the five compositions of TP conjugates, TP-2 system mediated a higher β-galactosidase gene expression than PEI system in Jurkat cell which was known to express elevated numbers of transferrin receptors. From the results of the cell viability based on MTT assay, TP conjugates showed lower cytotoxicity compared with the PEI system. We expect that the TP conjugate can be used efficiently as a non-viral gene delivery vector.

Key words: Transferrin, Polyethylenimine, Receptor-mediated, Gene Delivery, Conjugates

INTRODUCTION

Non-viral gene delivery systems such as lipoplexes, polyplexes and nanoparticles have been attracted, because these systems have advantages of safety, low cost, stability, ease of manufacturing and high flexibility concerning the size of the delivered transgene (Gebhart and Kabanov, 2001; Oku *et al.*, 2001; Remy *et al.*, 1998). The non-viral vector for systemic gene delivery should condense DNA and form a stable and small complex to facilitate diffusion, extravasation and internalization to avoid DNA sequestration and degradation in acidic vesicles (Erbacher *et al.*, 1999). Polyethylenimine (PEI) has been shown to be an efficient non-viral gene delivery vector both *in vitro* and *in vivo*. The high transfection efficiency of PEI is based on the fact that lysosomes would swell and burst due to the osmolarity changes on fusion with PEI-containing endosomes. But, the PEI has disadvantages as poor delivery to the target tissue or cells, toxicity at higher concentrations, and limited efficiency of delivery (Ahn *et al.*, 2002; Petersen *et al.*, 2002). In order to increase transfection efficiency and to add target specificity, the receptor-mediated cellular uptake has been developed by incorporating cell-binding ligands into the transfection complexes. Generally, the utilization of ligand-receptor binding for uptake into a target cell is dependent on the level of the receptor on the target cells and the affinity of ligand-receptor binding (Kircheis *et al.*, 2001). The design of targeted gene transfer systems is based on DNA/ligand-polycation complexes in order to achieve specific binding and internalization by receptor-mediated endocytosis (Ogris and Wagner, 2002). A variety of targeting ligands have been attached covalently or non-covalently to polycations such as epithelial growth factor (EGF)-conjugated PEI (Blessing *et al.*, 2001), folate-conjugated PEI (Guo and Lee, 1999), galactose-PEI (Morimoto *et al.*, 2003), galactosilated chitosan (Kim *et al.*, 2004), transferrin-polylysine (Wagner *et al.*, 1991), transferrin-protamine conjugates (Zenke *et al.*, 1990), transferrin-

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peptide nucleic acid (Liang *et al.*, 2000) to myogenic cell and PEI-polyethylene glycol-RGD peptide for angiogenic endothelial cell targeting (Suh *et al.*, 2002).

In this study, we have incorporated the transferrin into the PEI/DNA complex by chemical coupling to combine the high gene transfer efficacy of PEI/DNA complexes with the target-specific mechanism of receptor mediated uptake. Transferrin is a monomeric serum glycoprotein (Mw ~80,000 Da), and can bind up to two Fe^{3+} atoms for delivery to vertebrate cells through receptor-mediated endocytosis. Transferrin receptors are expressed in all nucleated cells in the body, and especially, in the proliferation and malignant cells (Citores *et al.*, 2002). Therefore, transferrin receptors are ideal targets for cancer therapy, and the transfection efficiency to cancer cells can be improved by conjugating transferrin with PEI. We synthesized and evaluated transferrin-PEI (TP) for transferrin receptor-mediated gene delivery that can target selectively on the cancer cells. The physical properties including size and zeta potential of TP/DNA complex and the gene expression in various tumor cells such as human acute T-cell leukemia (Jurkat) cells and human cervix epithelial carcinoma (HeLa) cells were described. And the optimized ratio of transferrin to PEI was determined to maximize the transfection efficiency and cell viability.

MATERIALS AND METHODS

Materials

Branched PEI (25 kDa) was purchased from Aldrich (St. Louis, MO, U.S.A.). Holo-transferrin (iron-saturated), 2,4,6-trinitrobenzene sulfonic acid (TNBS), 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), *N*-(2-hydroxyethyl) piperazine-*N*'-(2-ethanesulfonic acid) (Hepes), ethidium bromide (EtBr) and agarose were purchased from Sigma (St. Louis, MO, U.S.A.). Plasmid pCMVβ-gal (Clontech, Palo Alto, CA, U.S.A.) was isolated and purified using QIAfilter Midi Kits (Qiagen, Hilden, Germany). Plasmid pCMVβ-gal assay kit was purchased from Promega (Madison, WI, U.S.A.).

Synthesis of TP conjugates

Transferrin was dissolved in 30 mM cold sodium acetate buffer solution at pH 5.0, and then, mixed with 30 mM cold sodium periodate solution with keeping on ice for 90 minutes in dark place. The oxidized transferrin solution was added to PEI solution with vigorous mixing at room temperature. After 30 minutes, sodium cyanoborohydride (1 mg per 10 mg transferrin) was added to reaction mixture with four portions at 1 hour interval. The reaction mixture was incubated for 20 h at room temperature. The resulting TP conjugate was dialyzed for 1 day using spectra/por® membrane (MWCO=100,000) against 2 L HBS (150 mM NaCl in 20 mM Hepes, pH 7.3), and subsequently dialyzed against distilled water for 1 day. After dialysis, TP conjugate was freeze-dried and stored at -20°C until use.

Characterization of TP conjugates by FT-IR and gel permeation chromatography

IR spectra were obtained by FT-IR spectrometer (Nicolet 520, Nicolet Instrument, U.S.A.) using a potassium bromide pellet. Molecular weight and molecular weight distribution were measured by gel permeation chromatography (GPC) system (DAWN EOS, U.S.A.) using GPC columns (SB-803HQ, SB-802.5HQ). Purified water with 0.5 M ammonium acetate was used as eluant at 25°C. Samples were eluted at a flow rate of 0.5 mL/min. Molecular weight and molecular weight distribution of samples were calibrated with polystyrene standards (Waters, U.S.A.).

Analysis of the free amine contents

Quantity of the free amine in TP conjugates was determined by TNBS method (Snyder and Sobocinski, 1975). Briefly, 30 μg/mL TP conjugate solution was diluted with 0.1 M sodium tetraborate at pH 9.3 and adjusted the final volume as 1 mL. After mixing TNBS solution (0:03 M) with TP conjugate solutions and vortexing, the reaction mixture was incubated for 30 min at room temperature. The concentration of PEI was determined using standard curve at the ranges of 2 to 10 μg/mL. The absorbance at 420 nm was measured by UV spectrometer.

Agarose gel retardation assay

Gel retardation assay was used to determine the ability to condense DNA. The pCMVβ-gal plasmid was mixed with TP conjugate at selected N/P ratios. The TP/DNA complexes were incubated at room temperature for 30 min and then subjected to electrophoresis on 1% agarose gel containing 10 μg/mL ethidium bromide at 100 V for 45 min in Tris-acetate-EDTA buffer. Bands corresponding to plasmid DNA (pDNA) were detected by UV-illuminator.

Fluorescence spectroscopy measurement

For fluorescence measurement of EtBr intercalation, 20 μg/mL of pDNA was mixed with TP in various N/P ratio. The complex was diluted with distilled water to a final volume of 2 mL in each cell. After 30 min for incubation, the mixture was stained with 0.4 μg/mL EtBr. Quantification of EtBr intercalation into DNA base pairs was determined using spectrofluorometer (FP-6200, Jasco, Japan). The emission spectra were determined with excitation at 510 nm.

Measurement of particle size and zeta potential

The particle size and zeta potential of TP/DNA complex

were measured by an electrophoretic light scattering analyzer (ELS-8000, Otsuka, Japan). TP/DNA complex prepared at N/P ratio of 7 was diluted properly with distilled water and the determination of size and zeta potential was performed after incubation for 30 min.

Transfection experiments

Two kinds of cells were tested in order to determine the transfection and cell viability. For transfection experiments, Jurkat cells were seeded in 12-well plates just before the transfection (5×10^5 cells in 1 mL serum-free RPMI media). HeLa cells were seeded at a density of 2×10^5 cells/well in 1 mL of Dulbecco's modified Eagle's medium (DMEM) in 12-well plates and incubated for 24 h prior to the transfection. Cells were washed with serum-free medium and TP/DNA complexes were dropped into the cells and incubated for 4 h at 37°C in 5% CO_2 incubator. Medium was changed to serum containing RPMI 1640 or DMEM to remove the unbound complexes. After further culture for 44 h, cells were lysed in lysis buffer for 30 min, and cell debris was removed by centrifugation at 17,000 rpm at 4°C. The supernatant was transferred into the 96-well plates and added 50 μL of assay buffer containing β-mercaptoethanol and *o*-nitrophenyl β-D-galacto-pyranoside solution. After incubation for 30 min at 37 °C, the reaction was stopped by adding 1 M sodium carbonate (150 mL/well). The absorbance at 420 nm was measured by ELISA microplate reader (ELX808, Bio-TEK, U.S.A.).

Cell viability

Cytotoxicity of the TP/DNA complexes was determined by MTT assay in Jurkat cells. After the transfection, the growth medium was removed and replaced with the appropriate 950 μL fresh medium, and then 50 μL of 2 mg/mL MTT solution was added to each well. After incubation for 4 h at 37°C, each well was washed with PBS and formazan crystal produced by live cells was dissolved in 300 μL of dimethylsulfoxide. The absorbance was measured at 540 nm by ELISA microplate reader.

RESULTS

Synthesis and characterization of TP conjugates

TP conjugates with five compositions were synthesized by periodate oxidation method. Table I shows the feeding amount of transferrin and PEI for synthesis. Constant quantity of PEI (2.5 μmole) was mixed with various amounts of transferrin. Synthesized TP conjugates were confirmed by FT-IR spectroscopy and GPC. FT-IR intensities of PEI peaks at 1465 (CH_2 bending) and 3428 cm^{-1} (NH stretching) were decreased with increasing amount of transferrin, as shown in Fig. 1. But, the peak levels at 1542 (NH bending) and 1650 cm^{-1} (C=N) were increased with the increase of transferrin.

Table I. Compositions and molecular weight of TP conjugates (mean ± S.D., n=3)

Sample	Feeding amount		Molecular weight		
	Transferrin (imole)	PEI (imole)	Mn	Mw	Polydispersity
TP-1	0.0625	2.5	[a]N.D.	[a]N.D.	[a]N.D.
TP-2	0.125	2.5	107,800	110,400	1.024 ± 0.309
TP-3	0.25	2.5	178,700	183,200	1.025 ± 0.062
TP-4	0.625	2.5	196,200	206,100	1.050 ± 0.073
TP-5	1.25	2.5	221,100	237,800	1.075 ± 0.017
Tf			129,400	131,500	1.016 ± 0.032
PEI			46,580	61,580	1.322 ± 0.165

[a] N.D. : Not determined.

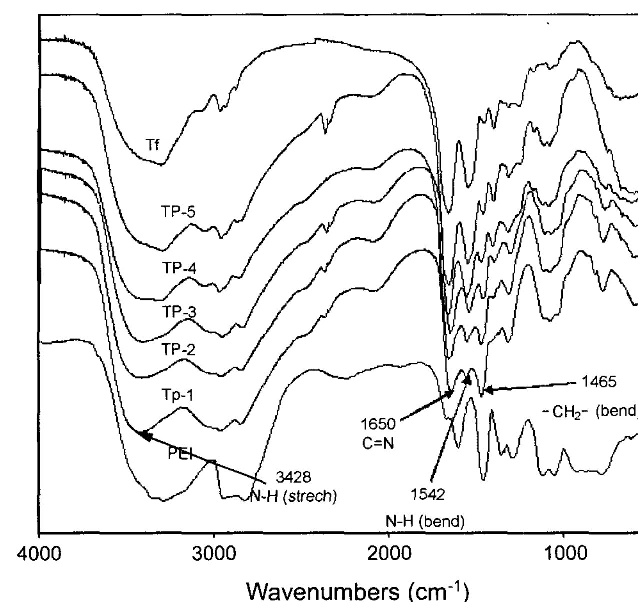


Fig. 1. FT-IR spectra of TP conjugates

Molecular weights of TP conjugate determined by GPC were ranged between 100 kDa and 240 kDa, as shown in Table I. The average molecular weight of TP conjugate increased as the amount of transferrin increased. Free amine of PEI is able to condense and deliver DNA. Fig. 2 shows the free amine content and transferrin content in TP conjugates. While transferrin content was increased, free amine content was decreased with the increase of transferrin.

Agarose gel electrophoresis

Various N/P ratios of TP/DNA complexes were obtained with a constant amount of pDNA, and the complexes were electrophoresed through agarose gels. The N/P ratio is defined as the number of protonatable nitrogen atoms per DNA phosphate group, and is frequently used

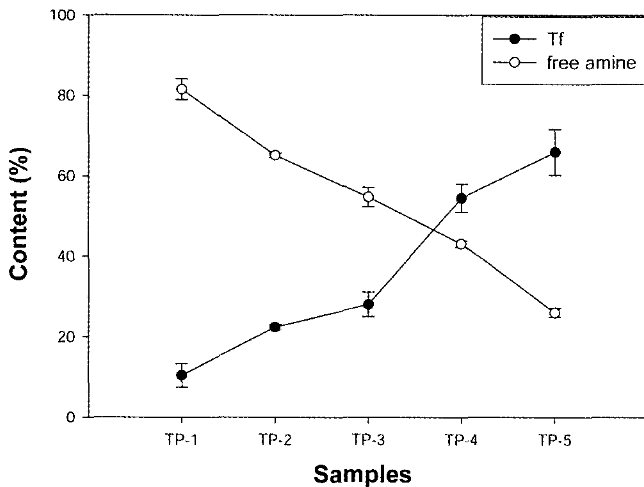


Fig. 2. Amounts of free amine and transferrin in TP conjugates

as a measure of the charge balance in polycation/DNA complexes. The efficiency of interactions between polycation and pDNA was evaluated by determining the amount of conjugate required to retard the migration of DNA. Agarose gel electrophoresis was performed with the PEI/DNA and TP/DNA complexes at N/P ratio of 3, 5, 7, and 10. As shown in Fig. 3, the complete complexation of TP-1 to TP-3 appeared above the N/P ratio of 5, TP-4 above the N/P ratio of 7, and TP-5 above the N/P ratio of 10, respectively. PEI was completely complexed with DNA above N/P ratio of 3.

Fluorescence spectroscopy measurement

The reduction of fluorescence after the addition of EtBr to polycation/DNA complexes can be regarded as a measurement for the efficiency of complex formation of the polycation (Merdan *et al.*, 2003). Fig. 4 showed the fluorescence emission spectra of EtBr, DNA/EtBr, and EtBr with TP-4/DNA complexes. The relative fluorescence intensity decreased with increasing N/P ratio due to the inaccessibility of EtBr for DNA when complexed with PEIs. At a low N/P ratio, however, a condensed plasmid particle cannot be formed efficiently, even with the presence of PEI (Liang *et al.*, 2000). In the same N/P ratio, TP conjugate with lower transferrin quantity had a better ability to condense pDNA (Data not shown).

Particle size and zeta potential of TP/DNA complexes

The mean particle size is a crucial factor which affects the diffusion of the gene vector to the target cell in the tissues (Rudolph *et al.*, 2002). The particle size of TP/DNA complexes was measured at N/P ratio of 7, as shown in Table II. The diameter of TP/DNA complexes was ranged between 210 and 250 nm in the all TP systems. Theoretically, this grade of size might not have difficulty in systematic application (Kircheis *et al.*, 1999).

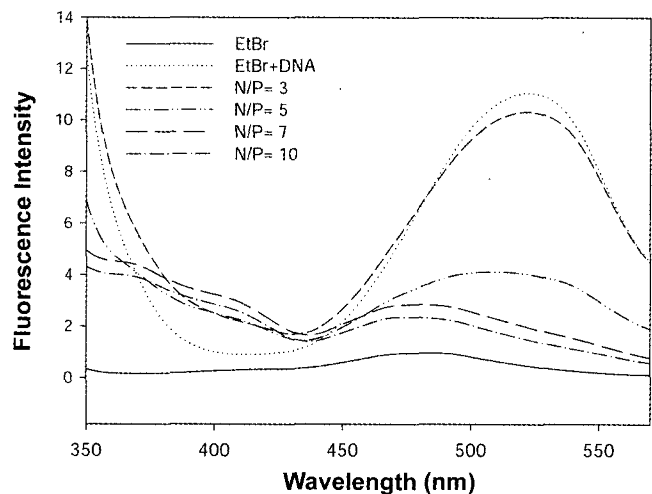


Fig. 4. Fluorescence emission spectra of EtBr, DNA/EtBr, and EtBr with TP-4/DNA complexes

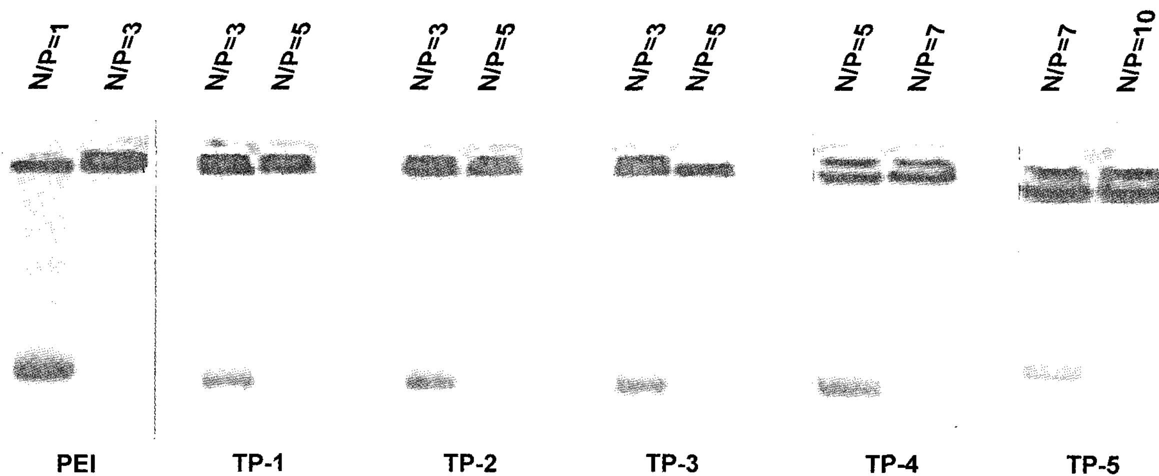


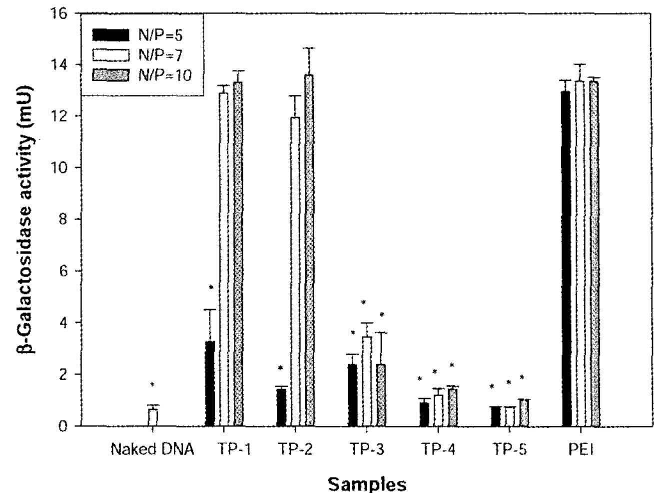
Fig. 3. Agarose gel electrophoresis of PEI/DNA and TP/DNA (pCMVβ-gal) complexes at various N/P ratios

The surface charge of complexes is an important factor for gene delivery. With the increasing amount of transferrin, the zeta potential of TP/DNA complexes was decreased because the negatively charged transferrin can compensate for the positive charges of PEI. TP/DNA complexes except TP-5 system showed the positive zeta potential, as shown in Table II.

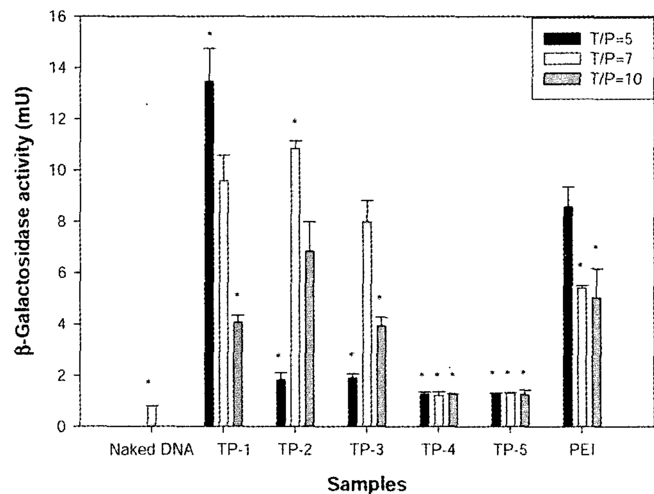
Transfection efficiency of TP conjugates

The *in vitro* transfection ability of TP/DNA complexes was evaluated from two different cell lines of HeLa cells and Jurkat cells using pCMV/β-gal as a reporter system. The same protocol was used for both TP and PEI with the individual N/P ratios. In HeLa cells (Fig. 5a), TP conjugates showed a comparable transfection efficiency to PEI. But, in Jurkat cells (Fig. 5b), TP-1 and TP-2 systems exhibited a significant increase ($p<0.01$) of transfection efficiency. As the quantity of transferrin was increased, the transfection efficiency was decreased. In the same composition, the transfection efficiency was dependent on the N/P ratio. Above N/P ratio of 7, transfection efficiency on Jurkat cell was decreased.

Receptor-mediated uptake can be demonstrated by the competition with an excess of the free ligand (Kircheis *et al.*, 2001). As shown in Fig. 6, free transferrin reduced the transfection activity of TP-2/DNA complexes as the function of the concentration of transferrin in Jurkat cell line, which is known to express elevated numbers of transferrin receptors (Pouton, 1999). Meanwhile, free transferrin had little effect on transfection efficiency of PEI. These data showed that receptor-mediated endocytosis of TP/DNA polyplexes can be competitively blocked by the excess free transferrin. The blocking by the addition of free transferrin was thus demonstrated that the TP/DNA complexes required the binding to the transferrin receptor on the target cells for gene delivery (Kircheis *et al.*, 1997).



(a)



(b)

Fig. 5. Transfection efficiency of TP/DNA complexes in (a) HeLa cell and (b) Jurkat cell (n=6). *$p<0.01$ compared with PEI (N/P=5).

Cell viability of TP/DNA complexes

Cell viability of TP/DNA complexes was evaluated using an MTT colorimetric assay. The cytotoxicity of cationic polymers was thought to be a result of membrane damaging effects, and the increase in the N/P ratio could be harmful to the cells since the increasing positive charge might be irritating or damaging to cellular membranes (Godbey *et al.*, 1999). Cell viability of TP/DNA complex on Jurkat cell was higher than that of PEI/DNA complex, as shown in Fig. 7. PEI is known to be toxic due to the membrane damaging effects of PEI (Godbey *et al.*, 1999). By conjugating transferrin with PEI, cytotoxicity of TP/DNA complex was reduced. In the same composition, the cell viability was decreased with increasing of N/P ratio. Cell viability on HeLa cell showed similar results with Jurkat cell (Data not shown).

Table II. Particle size and zeta potential of TP/DNA (N/P=7) complexes (mean ± S.D., n=3)

Sample	Particle size (nm)	Zeta potential (mV)
TP-1	237.9 ± 12.41	15.91 ± 0.30
TP-2	251.2 ± 25.22	12.86 ± 0.13
TP-3	235.2 ± 31.62	9.04 ± 0.11
TP-4	222.9 ± 15.40	3.43 ± 0.13
TP-5	214.7 ± 24.93	-19.50 ± 0.01
PEI	203.7 ± 31.78	25.53 ± 1.18

DISCUSSION

As the transferrin was non-immunogenic and could be conjugated without losing the biological activity, it can be

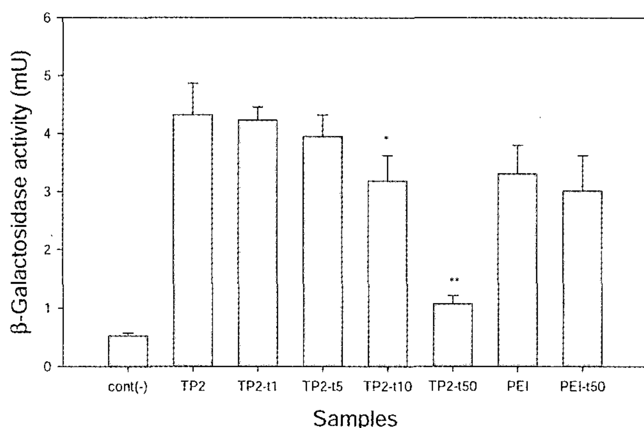


Fig. 6. Comparison of transfection efficiency of TP-2 conjugate and PEI following the pretreatment of transferrin (n=3). The notations of t1, t5, t10 and t50 represent the pretreated concentration of transferrin having 0.125, 0.625, 1.25 and 6.25 mM, respectively. *$p<0.05$ compared with TP-2 and ** $p<0.01$ compared with TP-2.

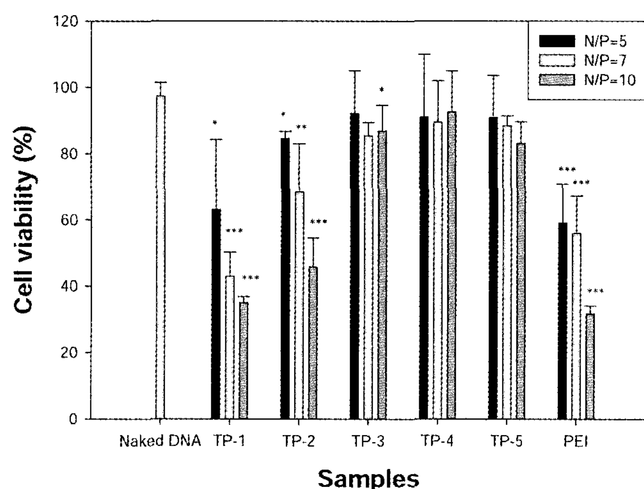


Fig. 7. Cell viability of TP/DNA complexes by MTT assay in Jurkat cell (n=6). $p < 0.05$ compared with naked DNA, **$p<0.01$ compared with naked DNA and ***$p<0.001$ compared with naked DNA.

conjugated with PEI in order to apply to the receptor-mediated gene delivery. PEI contains primary amino-groups which may be conjugated with ligand either directly or *via* spacer (Guo and Lee, 1999). Coupling of transferrin to PEI can be easily done by oxidation of the carbohydrate group of transferrin by sodium periodate followed by the reaction with the primary amino groups of PEI (Kircheis *et al.*, 2001). Five kinds of TP conjugates were synthesized in order to evaluate the effect of transferrin amounts in TP on the transfection efficiency.

The extent of the DNA condensation with TP was determined by measuring the change of the fluorescence intensity of EtBr, DNA intercalating agent. The condensation of polyanionic DNA by free amine of TP conjugates was important because of the prerequisite for gene delivery employing polycations. Condensation of DNA was increased as the ratio of transferrin in TP and the N/P ratio were increased, as shown in Fig. 4. This result was consistent with the electrophoresis result (Fig. 3).

The positive polycations will interact directly with the cell surface of many cell types due to the presence of negatively charged cell-surface molecules like proteoglycans (Ogris and Wagner, 2002). Due to the positive charges of polycation at physiological conditions, they interact with the negatively charged pDNA and form complexes with different charges and sizes. Zeta potential of TP/DNA complexes decreased as the ratio of transferrin in TP increased as shown in Table II. TP-5 system having high fraction of transferrin showed the negative zeta potential because of the negative charge of transferrin (Ogris *et al.*, 1998) and the shielding effect of transferrin chain.

The N/P ratio of polyplexes reflects the overall positive to negative charge balance of the DNA complex. Polyplexes formed at higher N/P ratios are more positively charged; therefore, they can interact with the negatively charged cell surface *via* nonspecific charge interaction. For PEI/DNA complex, transfection activity was increased with an increase in N/P ratio. This could be due to the combination of an increase in the positive charge of the corresponding polyplexes and an increase of PEI content in the DNA polyplex, which leads to elevated cellular uptake and more efficient endosome release of the internalized polyplexes, respectively (Guo and Lee, 1999). But, TP system showed different features. As the N/P ratio was increased in TP system, the quantity of transferrin as well as PEI were increased. Increasing the number of transferrin units per polymer would increase the affinity for the receptors, but too many transferrin residues might reduce the ability of the polymer to interact with pDNA (Morimoto *et al.*, 2003). Therefore, the optimum N/P ratio should be considered in TP systems. The transfection efficiency was decreased as the ratio of transferrin in TP systems was increased, as shown Fig. 5. These phenomena were explained from that the bulky transferrin molecule can interfere with DNA condensation when low molecular weight PEIs are used (Ogris *et al.*, 2003). Therefore, the low transfection efficiency of TP-5 was interpreted due to the low condensation of DNA and the negative zeta potential.

To test whether the transferrin receptor was involved in transferrin-PEI-mediated gene transfer, a competition assay was performed. Addition of exogenous transferrin in cell culture medium during transfection of pCMV/β-gal did not interfere with PEI/DNA receptor-independent gene transfer (Fig. 6). However, competition of transferrin-containing DNA complexes with free transferrin (TP2-t50) decreased gene transfer efficacy down to nearly 75%. Competitive and comparative transfection experiments

strongly suggested the involvement of transferrin receptor in gene delivery with TP-based systems. These findings were consistent with other results such as EGF-PEI (Blessing *et al.*, 2001), folate-PEG-PEI (Guo and Lee, 1999), PEG-PEI-Fab (Merdan *et al.*, 2003), galactose-PEI (Morimoto *et al.*, 2003) and biotin-transferrin-peptide nucleic acid/PEI (Liang et al., 2000) systems.

It is well known that positive surface charge of the polyplexes could be responsible for the toxicity (Kircheis *et al.*, 1999), while neutral or slightly positive complexes, which cannot bind efficiently to the cells, were much less efficient at transfecting myoblasts (Liang *et al.*, 2000). Therefore, TP-1 and TP-2 system, which showed high transfection efficiency, exhibited higher cytotoxicity than TP-4 and TP-5.

CONCLUSION

Non-viral gene delivery carriers have been conjugated with diverse ligands that selectively target particular receptors on certain type of cells. This receptor-mediated gene transfer facilitates the internalization of DNA complex via a receptor-mediated endocytosis pathway. In this study, five kinds of TP conjugates were synthesized. TP conjugates showed significant transfection efficiency on Jurkat cells. It can be expected that the TP conjugate is useful for gene delivery to overcome the weak point of PEI. Furthermore, we expect that the TP conjugate can be used more efficiently as a non-viral vector *in vivo*.

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